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VOLUME 27 FEBRUARY, 1949 NUMBER 1

— SECTION E —

MEDICAL SCIENCES

Contents

Page

Factors Relating to the Quantitative Estimation of Low Levels of Diphtheria Antitoxin by the Rabbit Skin Test —*L. Greenberg and J. Gibbard* - - - - - - - - - 1

Diagnostic Use of Tetraethylammonium Chloride (Etamon) in Occlusive Arterial Disease of the Extremities—*J. Farr and J. Doupe* - - - - - - - - - - - - 4

Plasma Magnesium in Disorders of the Thyroid Gland —*J. B. R. Cosgrove and W. F. Perry* - - - - - - - 10

An Effect of Ill Health on the Excretion of 17-Ketosteroids —*William F. Perry* - - - - - - - - - - - - 14

Preservation of Acetylcholine in Human Cerebrospinal Fluid: A Simple Method for Clinical Use—*Donald B. Tower* - - 20

The Relationship Between Serum Alkaline Phosphatase and Dietary Essential Amino Acids—*Jules Tuba, Max M. Cantor, and A. Gerald Richards* - - - - - - - - - - - 25

The Preparation of Isinglass Solutions Suitable for Transfusion Purposes—*H. D. Bell* - - - - - - - - - - - 31

Protection by Killed Vole Bacillus Vaccine Against Experimental Tuberculosis in Guinea Pigs—*J. C. Paterson, D. W. Crombie, and J. C. Coles* - - - - - - - - - - - 37

The Mucopolysaccharides of Human Skin—*R. H. Pearce E. M. Watson* - - - - - - - - - -

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JOURNAL OF RESEARCH

VOLUME 27 APRIL, 1949 NUMBER 2

— SECTION E —

MEDICAL SCIENCES

Contents

Page

Hépatectomie Partielle et Résistance aux Brûlures. V. Variations de l'Acide Ascorbique et du Poids des Surrénales —*L. P. Dugal and A. DesMarais* - - - - - - - - 59

Lipids of the Nervous System During *in vitro* Degeneration —*A. C. Johnson, A. R. McNabb, and R. J. Rossiter* - - - 63

Some Factors Affecting the Auscultatory Measurement of Arterial Blood Pressures—*A. E. Thomson and J. Doupe* - - 72

Effect of Amytal on Skeletal Muscle—*Bernard E. Riedel and Mervyn J. Huston* - - - - - - - - - - - - 81

The Gastric Response of Man to an Acid Test Meal—*Gordon E. Bermak and J. Doupe* - - - - - - - - - - - - 90

The Reaction of Various Tissues to Implants of a Collagen Derivative—*Arthur F. Battista* - - - - - - - - 94

Acetylcholine and Neuronal Activity. I. Cholinesterase Patterns and Acetylcholine in the Cerebrospinal Fluids of Patients with Craniocerebral Trauma—*Donald B. Tower and Donald McEachern* - - - - - - - - - - - 105

Acetylcholine and Neuronal Activity. II. Acetylcholine and Cholinesterase Activity in the Cerebrospinal Fluids of Patients with Epilepsy—*Donald B. Tower and Donald McEachern* - - - - - - - - - - - - - - 120

The Content and Characterization of Cholinesterases in Human Cerebrospinal Fluids—*Donald B. Tower and Donald McEachern* - - - - - - - - - - - - - 132

Some Effects of Diisopropyl Fluorophosphate (DFP) and Fluoroacetate on the Central Nervous System—*Andrew Kelen and Donald McEachern* - - - - - - - - - - - - 146

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CANADIAN JOURNAL OF RESEARCH

VOLUME 27 JUNE, 1949 NUMBER 3

— SECTION E —

MEDICAL SCIENCES

Contents

Page

The Determination of Phospholipid Phosphorus—*J. M. R. Beveridge and S. E. Johnson* - - - - - - - - - 159

Agglutination of Erythrocytes Containing Sulphaemoglobin—Further Evidence of Specific Aggregation—*Kenneth W. McKerns and Orville F. Denstedt* - - - - - - - - 164

The Effect of Antitoxin on Experimental Infection with *C. novyi* (*oedematiens*) in Guinea Pigs, With a Note on Active Immunization—*G. G. Waters and P. J. Moloney* - - - 171

Studies on the Effect of Some Aromatic Amidines and Other Chemical Compounds on the Growth of Influenza Virus in the Embryonated Egg—*Laurella McClelland and C. E. van Rooyen* - - - - - - - - - - - - - 177

An Electric Cauter as a Sterility Aid in Inoculation of Eggs —*N. A. Labzoffsky* - - - - - - - - - - - - 186

A Simple Gas Analyzer—*F. E. J. Fry. With an Appendix by A. C. Burton and O. G. Edholm* - - - - - - - - - 188

The Lipidolytic Enzymes of Larvae of *Galleria mellonella* —*Edith Mankiewicz* - - - - - - - - - - - 195

The Relationship of Serum Phosphatases to Sex Hormones —*Jules Tuba, Donald B. Baker, and Max M. Cantor* - - - 202

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VOLUME 27 AUGUST, 1949 NUMBER 4

— SECTION E —

MEDICAL SCIENCES

Contents

Page

Blood and Spinal Fluid Guanidine in Epilepsy—*K. A. C. Elliott and Nora Henderson* - - - - - - - - - - - 211

On the ABO Gene and Rh Chromosome Distribution in the White Population of Manitoba—*B. Chown, R. F. Peterson, Marion Lewis, and Ann Hall* - - - - - - - - - 214

On Ergothioneine: Its Preparation from Blood and Ergot—*G. Hunter, G. D. Molnar, and N. J. Wight* - - - - - 226

The Determination of Ergothioneine in Simple Solution and in Blood—*G. Hunter* - - - - - - - - - - - - 230

Note on the Ergothioneine Content of Ergot from Different Plant Hosts—*G. Hunter, S. G. Fushtey, and D. W. Gee* - - 240

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CANADIAN JOURNAL OF RESEARCH

The *Canadian Journal of Research* is issued in six sections, as follows:

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VOLUME 27 OCTOBER, 1949 NUMBER 5

— SECTION E —

MEDICAL SCIENCES

Contents

Page

The Effect of Liver Extract Preparation on Bone Marrow Explants—*K. E. Armbrust and H. D. Bell* - - - - - 245

Electron Microscopy of Typhus Rickettsiae—*C. E. van Rooyen and G. D. Scott* - - - - - - - - - - - - - 250

The Detection and Assay of Hyaluronidase by Means of Mucoid Streptococci—*R. G. E. Murray and R. H. Pearce* - 254

The Microcolorimetric Determination of Inorganic Phosphate in Plasma and Urine. I. Quantitative Investigation of Present Common Procedures. II. A Sensitive and Stable Method for Plasma, Serum, and Urine—*John R. Polley* - 265

The Effect of Iodoacetate on the Apparent Synthesis of Diphosphothiamine During the Estimation of the Cocarboxylase Activity of Blood Extract—*M. C. Blanchaer and L. H. Cohen* 275

Lipotropic Activity of Choline, Betaine, and Methionine in Ducklings—*R. Bernard and J. M. Demers* - - - - - 281

Phosphatase of Rabbit Polymorphonuclear Leucocytes—*D. M. Cram and R. J. Rossiter* - - - - - - - - - 290

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OTTAWA, CANADA

CANADIAN JOURNAL OF RESEARCH

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VOLUME 27 DECEMBER, 1949 NUMBER 6

— SECTION E —

MEDICAL SCIENCES

Contents

Page

Interfacial Precipitation of Cholesterol in Human and "Artificial" Biles—*Philip Fitz-James and Alan C. Burton* - 309

The Production of Acute Massive Hepatic Necrosis in Rats by Dietary Means—*M. R. Abell and J. M. R. Beveridge* - - 316

The Effect of Surgical Operations on the Excretion of Iodine, Corticosteroids, and Uric Acid—*W. F. Perry and J. P. Gemmell* 320

Cholinesterases of Cerebrospinal Fluid. Data for Normal Fluids and Fluids from Patients with Syphilis, Meningitis or Poliomyelitis—*K. G. Colling and R. J. Rossiter* - - - 327

Some Observations on Interference Between Neurotropic Viruses—*A. J. Rhodes and Marion Chapman* - - - - 341

Effets de l'Acide Ascorbique sur Plusieurs Variables Biologiques Normalement Affectées par le Froid—*M. Thérien, J. Leblanc, O. Héroux, and L. P. Dugal* - - - - - - - - - - 349

L'Action Anémiante de Deux Antagonistes de la Lysine: l'Hexahomosérine et l'Acide α-Aminoadipique—*Edouard Pagé, Rosaire Gingras, and Roger Gaudry* - - - - - - 364

Effects of Eserine and Acetylcholine on the Respiratory Centers and Hypoglossal Nuclei—*Frederick R. Miller* - - - - 374

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VOL. 27, SEC. E. FEBRUARY, 1949 NUMBER 1

FACTORS RELATING TO THE QUANTITATIVE ESTIMATION OF LOW LEVELS OF DIPHTHERIA ANTITOXIN BY THE RABBIT SKIN TEST[1]

BY L. GREENBERG[2] AND J. GIBBARD[3]

Abstract

A simple method for the quantitative determination of small amounts of diphtheria antitoxin is presented. Toxin of known strength is mixed with various dilutions of serum. These are held at room temperature for two hours and 0.1 cc. of each dilution is injected intracutaneously into the back of a suitably prepared rabbit. The resulting skin reactions are compared with reactions obtained with control mixtures, containing known amounts of antitoxin, injected at the same time. Between 200 and 300 tests may be performed on the back of a single rabbit.

Introduction

The classical method of Ehrlich for measuring diphtheria antitoxin is the standard procedure in all countries. This method, however, is only suitable for measuring solutions containing one or more units of antitoxin per cc. For the measurement of small amounts of antitoxin, namely fractions of one unit such as may be found in human sera, intracutaneous tests on guinea pigs and rabbits are used.

In all tests of this type the principles involved are the same. A known amount of toxin is mixed with varying dilutions of antitoxins. The mixtures are held for varying lengths of time and then injected into animals. The degree of the resulting reaction indicates the strength of the antitoxin. For an Ehrlich test, the test dose of toxin is the L+ dose, and this is defined as that amount of the toxin which, when mixed with one unit of antitoxin and injected into a normal guinea pig weighing 250 gm., will kill the guinea pig on the fourth day after the injection. For an intradermal test, the dose of toxin will be that amount of toxin, which, when mixed with a known amount of antitoxin, will upon subsequent intradermal injection into the back of a rabbit or guinea pig cause a definite area of erythema. The known strength of antitoxin used can be varied for the test at hand but is usually 1/250 unit or less per cc.

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[1] *Manuscript received October 6, 1948.*

Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa.

[2] *Bacteriologist.*

[3] *Chief.*

The method was originally devised with guinea pigs (4, 6) following Römers observation that diphtheria toxin produced a specific and characteristic lesion when injected experimentally into these animals.

The test was modified later for use with rabbits by Fraser (2, 3) and Jensen (5). The procedures described below are details of the method in current use at the Laboratory of Hygiene and are designed to test at a level of 1/250 unit of antitoxin. Although the method is not original it is presented because of its importance to both clinical and control laboratories and because of the number of requests by laboratory workers interested in this technique. Further, workers on this continent do not generally appreciate the large number of tests (over 300) that can be placed on the back of one animal.

General Procedure

I. *The Ingredients*

(*a*) *Toxin*

Only well stabilized toxins should be used. The toxin in use at the Laboratory of Hygiene was stabilized by dilution in a borate buffer* gelatin solution (pH 7.6), the method recommended by Moloney and Taylor (7, 8). A freshly prepared lot of diphtheria toxin was diluted to contain 2 L+ doses per cc. Experimentation has shown the intradermal test dose of toxin to be 1/250 of an L+ dose, contained in 1.0 cc. There has been no detectable change in potency over a period of eight years.

(*b*) *The Antitoxin*

An international standard reference antitoxin is used as a control. It is used in three strengths, 1/125, 1/250, and 1/500 unit per cc.

(*c*) *The Diluent*

Borate buffer saline (pH 8.0) is used as the diluent.

(*d*) *The Test Animal*

Rabbits are used as the test animal. Prior to use, the back and flanks of the animals are closely clipped with suitable clippers. The backs are then marked off into approximately 2 cm. squares by means of a wooden applicator using a saturated solution of picric acid as the marking fluid.

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* *Adapted from Palitzsch's borax–boric acid mixture (1) the formula used for the preparation of this solution was:—*

(*a*)	*Sodium borate*	—	*13.1 gm.*
	Distilled water	—	*1 liter*
(*b*)	*Boric acid*	—	*12.4 gm.*
	NaCl	—	*2.9 gm.*
	Distilled water	—	*1 liter*
(*c*)	*Solution (a)*	—	*250 cc.*
	Solution (b)	—	*750 cc.*

Adjust to pH 7.6 using Solutions (a) and (b)

Dilute Solution (c) 1:4 using as diluent a 0.85% saline solution containing 0.02% gelatin. Readjust pH to 7.6 using Solutions (a) and (b).

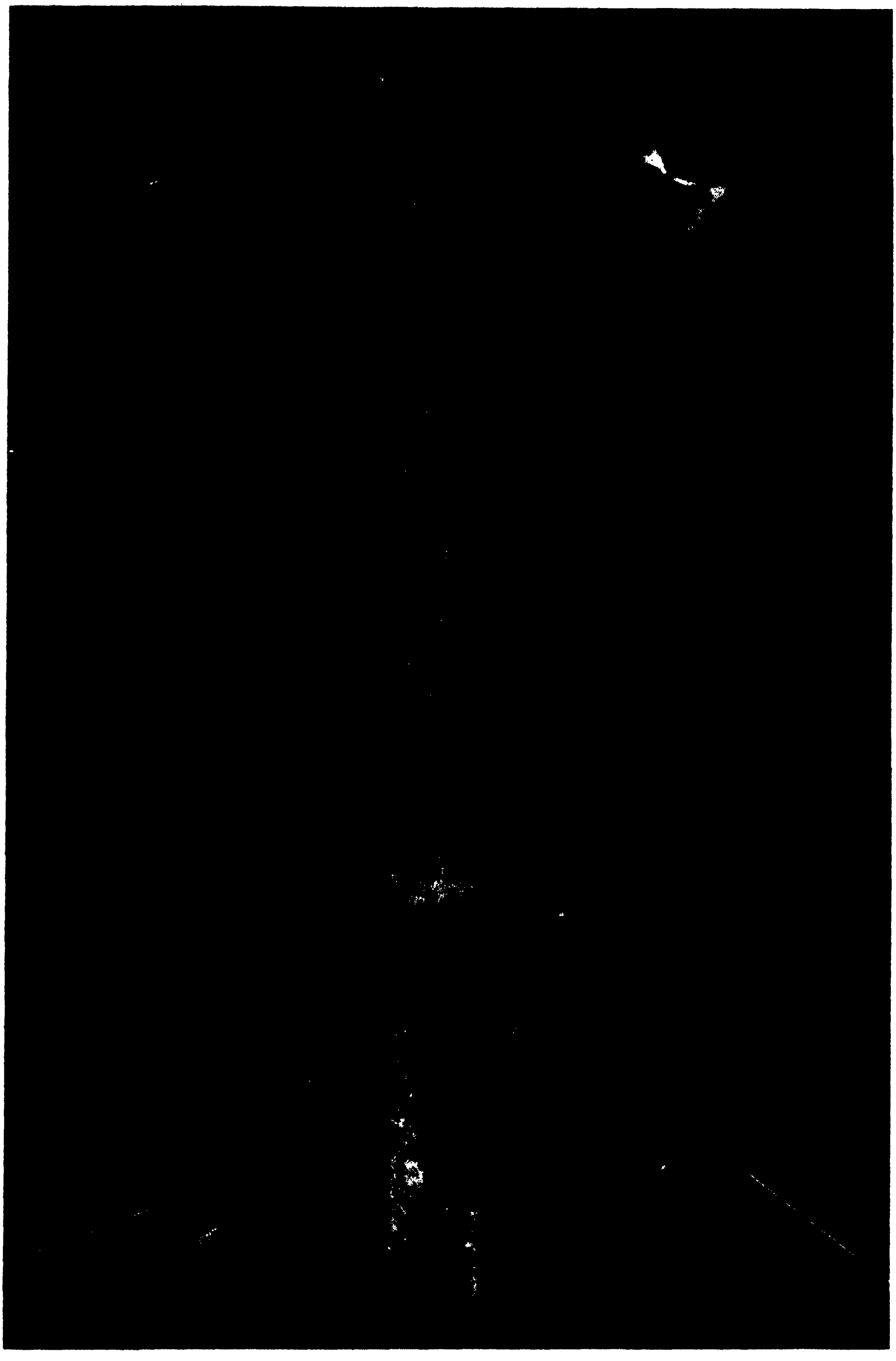

FIG. 1. *A typical rabbit skin test four days after inoculation.*

II. *Technique of Test*

The test dose of toxin is mixed with the same volume of dilutions of the solution to be tested, e.g., undiluted, 1 : 2 dilution, 1 : 4 dilution, etc. The mixtures are held at room temperature for two hours and then 0.1 cc. of each mixture is injected intracutaneously into designated squares on the back of the rabbit prepared as above. To serve as controls for the toxin and also to act as sources of comparison, three control mixtures containing 1/125, 1/250, and 1/500 unit of known antitoxin per cc. respectively are prepared at the same time as the above and injected intracutaneously into several designated regions on the back of the same animal. Three days later the individual skin reactions are measured and recorded. The strength of the unknown solution is measured by direct comparison with the known antitoxin controls. In a properly controlled test, the mixture containing 1/125 unit of antitoxin should leave no visible reaction, the mixture containing 1/250 unit should result in a mild erythema of between 4 to 8 mm. and the mixture with 1/500 unit should give a slightly heavier erythema of between 8 to 12 mm. The test dose of toxin, when injected alone, should cause an erythematous area of 10 to 15 mm. with some necrosis. The number of squares on the back of an animal will be influenced by two factors—the strength of the test toxin used, and the size of the rabbit. Jensen, testing at the 1/3000 antitoxin level, puts over 300 squares on one rabbit. At the Laboratory of Hygiene, where the work is generally with a level of 1/250 and 1/500 antitoxin units, up to 250 squares can be inoculated on one rabbit. The accompanying photograph illustrates a typical test on a rabbit four days after the inoculation.

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DIAGNOSTIC USE OF TETRAETHYLAMMONIUM CHLORIDE (ETAMON) IN OCCLUSIVE ARTERIAL DISEASE OF THE EXTREMITIES[1]

By J. Farr and J. Doupe

Abstract

Using the temperature of the digits to measure vasodilation, the dilating effect of tetraethylammonium chloride (Etamon) was compared to that of thermal stimulation. It was found in three normal subjects and 11 patients that doses of 300 to 500 mgm. administered intravenously never induced a higher skin temperature than did body heating and the mean maximum temperature was 5° to 6° C. lower. Because of the hypotensive effect of the drug higher doses were not used. It was concluded that Etamon was not a reliable agent for testing the vasodilatation potential of the peripheral circulation.

Introduction

In 1945 Acheson and Moe (1) described the paralytic effect of the tetraethylammonium salts upon the ganglia of the autonomic system and in later reports they and others indicated that tetraethylammonium chloride, i.e. Etamon, could be used to determine the amount of vasodilatation possible in patients with organic arterial disease of the extremities. In 1947 Coller *et al.* (3) reported further results and claimed that it was superior to any other method of vasodilatation. However, DeBakey (4) in discussing their paper stated that he had not formed a favorable opinion of the test and Boyd *et al.* (2), using the bromide salt, have contributed evidence supporting this view.

The present investigation was undertaken because of the importance of establishing whether or not such a simple procedure as an intravenous injection could replace the established methods that assist in the evaluation of the degree of organic arterial obstruction in an extremity. Reflex vasodilatation and sympathetic block are the two methods most commonly employed. Because the latter is to a great extent dependent upon the skill of the operator and therefore subject to technical error, reflex vasodilatation was used as the standard to which the effects of Etamon were compared.

Methods

Skin temperatures were used as an index of vasodilatation. They were recorded by means of copper–constantan (gauge 34) couples in contact with the pads of the exposed digits and held in place by narrow strips of adhesive tape on the dorsum of the second phalanx. Temperatures were read to 0.1° C. and were recorded at two to three minute intervals from the first and fourth digits of each foot and from the right index finger. Rectal temperatures were taken by a special thermocouple and read to 0.01° C.

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[1] *Manuscript received November 12, 1948.*

Contribution from the Departments of Surgery and Medical Research of the University of Manitoba and the Winnipeg General Hospital, Winnipeg, Man.

The subjects reclined in a constant temperature room (21° to 23° C.) and were clad in pyjamas. To provide a base line, recordings were made for 15 to 30 min. before a test. In approximately half of the cases Etamon was used prior to reflex vasodilatation and in the others the order was reversed. Between the tests sufficient time was allowed for skin cooling to occur and in some cases it was necessary to counteract the effects of reflex vasodilatation by immersing the left arm in cold water for a short time. As recommended by Coller *et al.* (3), Etamon was injected intravenously in doses of 300 and 500 mgm. in 40 to 60 sec. Within these limits advanced age, debility, and small stature were used as indications for the lower doses. Blood pressure and pulse rates were recorded at three minute intervals just prior to and for 15 min. following the injection.

Reflex vasodilatation was induced according to the method of Gibbon and Landis (5) by immersing the left arm in water at 44° C. and the heating effect was augmented by covering the trunk and proximal parts of the extremities with blankets. The subjects consisted of three normal young men and 11 patients with various types of vascular disease. These are detailed in Table I.

Results

The results of two experiments are illustrated in Figs. 1 and 2 to show the type of records used as a source for the data summarized in Table I. In

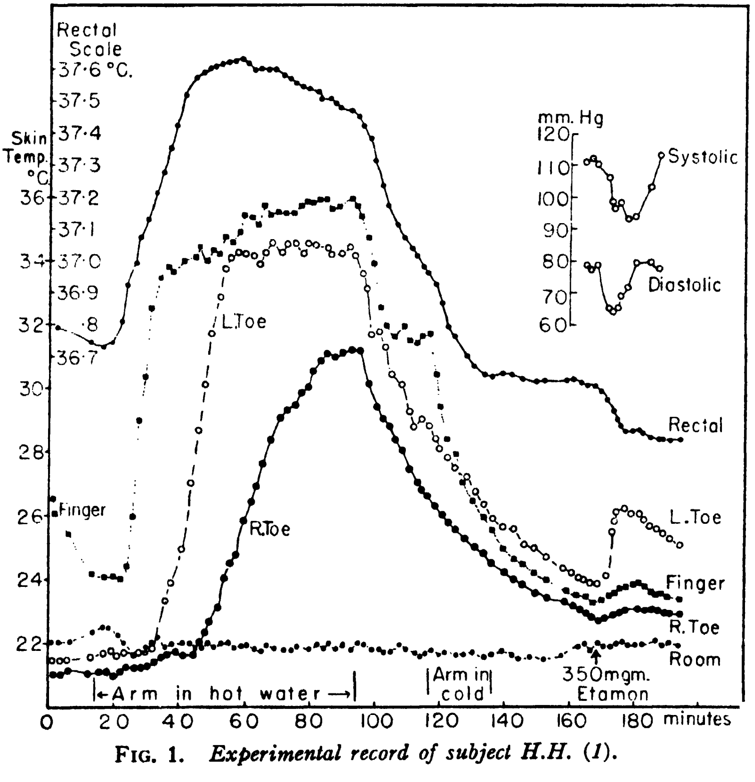

FIG. 1. *Experimental record of subject H.H. (1).*

TABLE I

EFFECT OF ETAMON CONTRASTED TO REFLEX VASODILATATION

Subject	Diagnosis	Sex	Age	Dosage		B.P., mm. Hg		Finger temp., °C.			Rt. toe temp., °C.			Lt. toe temp., °C.		
				Mgm.	Mgm./kgm.	Before Etamon	Fall	Re-flex	Eta-mon	Diff.	Re-flex	Eta-mon	Diff.	Re-flex	Eta-mon	Diff.
R.L.	Livedo reticularis	F	19	425	8.8	105/ 62	20/ 2	36	33*	3	37	34*	3	37	35*	2
A.R. 1	Normal	M	22	400	4.4	130/ 70	20/ 10	37	24	13	35	21	14	35	21	14
A.R. 2	Normal	M	22	500	5.1	116/ 85	8/ 15	35	24*	11	35	32*	3	35	32*	3
B.H. 1	Normal	M	25	400	6.2	122/ 62	0/-14	35	32	3	35	29	6	35	28	7
B.H. 2	Normal	M	25	400	6.2	120/ 75	10/ 15	35	32	3	35	32	3	35	33	2
B.H. 3	Normal	M	25	400	6.2	120/ 70	10/ 8	35	35	0	34	34	1	35	34	1
D.P.	Hypertension	M	30	500	5.1	235/155	55/ 5	36	35	1	34	29	5	34	29	5
N.M.	Thromboangiitis	M	32	350	6.0	160/ 70	50/ 20	36	32	4	24	24	0	36	24	12
J.D.	Normal	M	38	400	5.5	105/ 80	10/ 5	36	29*	7	36	34*	2	36	34*	2
E.L.	Hypertension	F	55	350	5.3	230/130	115/ 45	36	25	11	33	31	2	33	26	7
H.H. 1	Thrombophlebitis	M	55	350	4.7	112/ 80	17/ 15	36	24	12	31	23	8	35	26	9
H.H. 2	Thrombophlebitis	M	55	400	5.5	122/ 80	24/ 14	35	27*	8	32	25	7	34	30*	4
J.McK.	Arteriosclerosis	M	57	300	5.2	140/ 75	48/ 15	36	23	13	24	22	2	24	22	2
A.N.	Thrombophlebitis	M	60	500	6.4	165/ 90	80/ 40	35	35	0	35	26	9	35	27	8
C.S. 1	Arteriosclerosis	M	63	400	5.3	150/ 85	32/ 20	36	31	5	23	21	2	34	33	1
C.S. 2	Arteriosclerosis	M	63	470	6.3	140/ 72	52/ 24	36	34	2	23	22	1	34	32	2
A.S.	Arteriosclerosis	M	63	355	5.0	112/ 75	47/ 65	37	22	15	30	20	10	34	21	13
G.H.	Arteriosclerosis	M	68	350	5.0	175/ 90	80/ 32	36	29*	7	34	31*	3	27	27	0
B.R.	Arteriosclerosis	M	77	350	4.7	128/ 80	33/ 20	36	34	2	35	28	7	35	24	11

* *Finger and toe effects of Etamon can be compared.*

this table the highest temperatures attained are stated and the subjects are listed in order of age. Omitted are the observations made on the fourth toes because they closely resembled those recorded for the great toes. It can be seen that in nearly every instance the level of skin temperature produced by reflex vasodilatation was greater than that caused by the intravenous injection of Etamon. The average maximum temperature of the finger with reflex vasodilatation was 6° C. greater than the average maximum temperature following Etamon. The corresponding difference in the right great toe was 5° C. and in the left great toe 6° C. Figs. 1 and 2 also show a fall in the rectal temperature following Etamon; the fall in temperature was present in all subjects with the exception of N.M. and averaged 0.2° C.

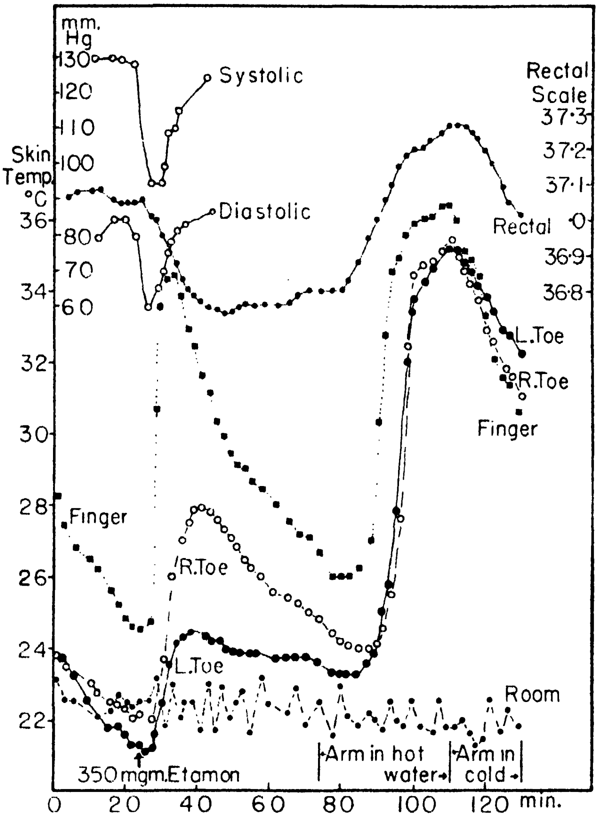

FIG. 2. *Experimental record of subject B.R.*

Table I shows that the discrepancy between the vasodilating action of heat and of Etamon differed greatly in the various subjects. This variation seemed to be due in part to the temperature of the digit when the Etamon was given and upon the size of the dose. To elucidate the influence of the initial temperature, three sets of observations with a constant dosage were made on subject B.H. In the first experiment reported on him in Table I, the initial temperature of the toes was 21° C., and in the second it was 25° C., and in the third 29° C. It will be seen that with an increase in initial temperature a higher maximal temperature was obtained with a consequent decrease in the discrepancy between the effect of Etamon and that of reflex vasodilatation.

The effect of dosage can also be seen in Table I. Subjects A.R., H.H., and C.S., who each received two different doses showed a more marked elevation with the larger dose. However, when comparing one individual with another the degree of response did not bear such strict relationship to the size of the dose.

In regard to the difference in the response of the upper and lower limbs, all the data in Table I are not directly applicable because of the effects of initial temperature or the presence of arterial obstruction. In those subjects in whom the initial temperatures of the fingers and toes were similar and in whom there was no organic vascular disease (marked by asterisk in Table I), it will be seen that Etamon caused a greater effect upon the toes than the fingers. This confirms the observations of Larsson and Frisk (6).

It can be seen in Table I that Etamon frequently caused a striking fall in blood pressure in the older age group. Excluding the subjects with hypertension, those under fifty had an average fall in blood pressure of 16/8 mm. Hg while for those above fifty the average fall was 46/27 although the latter received in most instances a smaller dose.

Discussion

The observations reported here indicate that Etamon is not a satisfactory vasodilating agent because the temperatures achieved did not bear a consistent relationship to the dilatation potential demonstrated to be present by the method of reflex vasodilatation. It is true that Larsson and Frisk (6) found Etamon as satisfactory as lumbar block, but they used doses of 10 mgm./kgm., which are approximately 50% larger than those used by Boyd *et al.* (2) and by Coller *et al.* (3).

In the three cases in which Larsson and Frisk (6) compared the action of these large doses to that of body heating, no superiority of either method was demonstrated. However, as they state that doses of less than 10 mgm./kgm. do not produce maximal dilatation, it is apparent that the present results are more in agreement with theirs than with those reported by Coller *et al.* (3). The discrepancies are difficult to explain but may be related to a difference either in the initial temperature of the limbs or to a difference in room temperature. It should also be noted that the illustrations of Coller *et al.* (3) show the greatest effect in 20 or 30 min. whereas the present observations and those of Larsson and Frisk showed the peak of the temperature rise to be achieved in less than 10 min.

Moreover, in the absence of a total autonomic block it is highly probable that the fall in rectal temperature and the fall in the blood pressure would induce peripheral vasoconstriction and thus tend to counteract the peripheral vasodilating action of Etamon. From this arises the necessity of using doses capable of producing complete blockade. Even 10 mgm./kgm. doses in the hands of Larsson and Frisk (6) did not produce results comparable to peripheral nerve blockade in four out of eight cases. The objection to doses of this size is the very marked fall in blood pressure, which is particularly pronounced in the arteriosclerotic group where vasodilation tests find their most important use. Because of the possibility that hypotensive interludes might produce serious complications in these subjects, it has not been considered advisable to explore the value of such large doses.

If such caution is warranted, it is apparent that the present results substantiate the opinion of DeBakey (4) and the findings of Boyd *et al.* (2) that Etamon is not a reliable agent for testing the vasodilatation potential of the peripheral circulation.

Acknowledgments

The equipment used in this study was furnished partly by the National Research Council of Canada and partly by the Manitoba Institute for the Advancement of Medical Education and Research. The Etamon was kindly supplied by Parke Davis Co. Mr. Arnold Rogers contributed valuable technical assistance.

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PLASMA MAGNESIUM IN DISORDERS OF THE THYROID GLAND[1]

By J. B. R. Cosgrove and W. F. Perry

Abstract

There is a conflict of evidence concerning the alterations of nondiffusible plasma magnesium in disorders of the thyroid gland. In this investigation it was found in 12 normal individuals, 9 patients with hyperthyroidism, 10 with myxedema, 6 with nontoxic goiter, and 14 patients with miscellaneous diseases, that the nondiffusible plasma magnesium was relatively constant in a wide range of conditions and was not altered even in patients with marked thyroid dysfunction.

Introduction

The total plasma magnesium can be separated into diffusible and nondiffusible fractions. While there is no reason to believe that the total value is altered in disorders of the thyroid gland, it has been claimed that the nondiffusible fraction is increased in hyperthyroidism and reduced in myxedema (4, 8, 9). However, normal values have also been reported in hyperthyroidism (2, 3). Because of the important role played by magnesium in many metabolic processes, this study was undertaken to determine whether or not alterations in the amount of nondiffusible magnesium occur in dysthyroid states.

Method

Two ml. of plasma, obtained by centrifugation of heparinized venous blood from fasting patients, was used for total magnesium determinations. To obtain the diffusible fraction 3 ml. was subjected to ultrafiltration. The value for the nondiffusible magnesium was obtained by subtracting the diffusible fraction from the total.

Magnesium was measured by the titan yellow method described by Kunkel *et al.* (6). The preparation of the standard solution was modified by preparing it with magnesium metal rather than magnesium sulphate septahydrate so as to avoid error due to the deliquescent properties of the salt. Nineteen mgm. of the metal was dissolved in 0.5 ml. of concentrated hydrochloric acid and diluted to 1 liter with distilled water. Owing to a change in the titan yellow solution it was found necessary to prepare a calibration curve daily. To determine the reliability of the method, eight magnesium determinations were made on the same plasma. A mean of 2.48 mgm. % was obtained with a range of 2.41 to 2.58 and a standard deviation of ±0.05%. In 10 recovery experiments, 19 μgm. of magnesium was added to 2 ml. of plasma. The mean recovery of added magnesium was 100% with a range of 96 to 106%.

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[1] *Manuscript received October 25, 1948.*

Contribution from the Departments of Medicine and Medical Research of the University of Manitoba and the Winnipeg General Hospital.

Utilizing an ultrafiltration apparatus of the type described by Nicholas (7), plasma was filtered through No. 300 wet cellophane using an air pressure of 125 lb. per sq. in. at a room temperature of 22° to 28° C. By placing the apparatus in an automatic shaker, 1 ml. of ultrafiltrate was obtained in approximately one hour. The performance of the filter was checked by testing all filtrates for protein with trichloroacetic acid. The reliability of the results was ascertained by showing that a second passage of an ultrafiltrate did not alter its concentration of magnesium, and in addition, 17 ultrafiltrates from the same sample of plasma had a mean magnesium value of 1.29 mgm. % with a range of only 1.18 to 1.38 and a standard deviation of ±0.05%. The concentration of magnesium in the ultrafiltrate was found not to be affected by varying the air pressure, soaking the cellophane in distilled water for varying periods, using oxalate instead of heparin as the anticoagulant, using serum instead of plasma, varying the amounts of plasma in the ultrafilter, using or not using the automatic shaker.

Five groups of subjects were studied. Those in the four abnormal groups were selected as having typical examples of their respective disorders. The diagnosis of the dysthyroid states was confirmed by basal metabolic readings and plasma protein-bound iodine determinations. The normal group consisted of six females and six males who were laboratory technicians or medical students with an age range of 20 to 30 years. The hyperthyroid group was made up of seven females and two males, ranging in age from 26 to 62, with a mean B.M.R. of +49% (range +29 to +78) and a mean protein-bound iodine value of 17.5 μgm. % (range 12.8 to 20.0). The nontoxic nodular thyroid group consisted of six females ranging in age from 30 to 79, with a mean B.M.R. of +3% (range −6 to +21) and a mean protein-bound iodine of 5.5 μgm.% (range 4.8 to 7.3). The hypothyroid group was composed of seven females and three males, varying in age from 18 to 72 years, with a mean B.M.R. of −35% (range −23 to −47) and a mean protein-bound iodine of 1.6 μgm. % (range 0.0 to 3.7 μgm.). The group of patients with miscellaneous diseases consisted of 10 females and 4 males with an age range of 6 to 71 years. The plasma protein-bound iodine was within the normal range (4 to 9 μgm. %). The B.M.R. was normal except in the cases of congestive heart failure and leukemia in which it was elevated. In the group were five patients with psychoneurosis and single examples of Paget's disease, cirrhosis of the liver, psychosis, infectious hepatitis, congestive heart failure, essential hypertension, carcinoma of the pancreas with jaundice, myelogenous leukemia, and diabetes mellitus.

Results

The levels of total plasma magnesium and its fractions in the various groups are shown in Table I. It is apparent that the nondiffusible fraction of the various groups showed no significant difference in either level or in

TABLE I

Plasma magnesium in various groups

Group	Number of cases	Mean total, mgm. % ± S.d. and range	Mean diffusible, mgm. % ± S.d. and range	Mean nondiffusible, mgm. % ± S.d. and range*	Mean % nondiffusible, ± S.d. and range**
Normal	12	2.60 ± 0.06 (2.50 to 2.71)	1.28 ± 0.12 (1.13 to 1.48)	1.30 ± 0.12 (1.11 to 1.50)	50.9 ± 4.6 (43.0 to 56.5)
Miscellaneous	14	2.66 ± 0.19 (2.33 to 2.98)	1.26 ± 0.12 (1.07 to 1.50)	1.13 ± 0.22 (1.01 to 1.80)	52.3 ± 5.7 (40.2 to 62.1)
Hyperthyroid	9	2.58 ± 0.19 (2.23 to 2.80)	1.32 ± 0.12 (1.14 to 1.55)	1.25 ± 0.19 (1.01 to 1.52)	48.3 ± 4.9 (41.8 to 57.1)
Nontoxic nodular thyroid	6	2.57 ± 0.14 (2.35 to 2.70)	1.24 ± 0.13 (1.08 to 1.44)	1.32 ± 0.11 (1.15 to 1.43)	51.4 ± 3.9 (46.4 to 55.7)
Hypothyroid	10	2.75 ± 0.19 (2.56 to 3.05)	1.32 ± 0.11 (1.20 to 1.54)	1.43 ± 0.12 (1.32 to 1.73)	52.1 ± 2.7 (47.4 to 56.6)

* *Difference between total and diffusible.*

** *Percentage of total.*

percentage relationship to the total. The values for the total plasma magnesium also were similar for the various groups, the highest being found in myxedema.

Discussion and Summary

In considering the above results it should be noted that the values for total plasma magnesium are slightly higher than those found by Kunkel *et al.* (6) who also used the titan yellow method. This may be due to the difference in standard solutions. However, the values reported here are well within the accepted range of 2 to 3 mgm. %. It is of interest that the values for total magnesium in the myxedematous group were slightly higher than in the normal group. This may bear some relationship to recent evidence that shows that a high total serum magnesium is associated with subnormal body temperature (10, 11, 12).

It is customary to express the value for nondiffusible magnesium as a percentage of the total. In the present investigation this was found consistently to be in the region of 50%. This value is at variance with previous results. Soffer *et al.* (8, 9) report for normals a mean of 14.5% (range 3.1 to 22.1), Cope and Wolff (3) found a mean of 37% (range 14 to 56), Dine and Lavietes (4) found a range of 14 to 31%, while Bissell (1) reports a mean of 30.4% (range 17 to 42). This difference in results must indicate some difference in technique or methods. The above authors used the method of Briggs (2) or some modification of it to determine the magnesium concentration. As this method necessitates the precipitation of calcium, there is a possibility that this precipitation may affect the subsequent determination of magnesium.

In the titan yellow method, calcium in the concentration found in human plasma does not affect the estimation of magnesium (5). This may be the explanation for the variance of results.

Whatever the correct explanation may be for the discrepancy between the present results and those of other investigators, the consistency of the results reported here gives confidence to the conclusion that neither the total nor the nondiffusible plasma magnesium is affected by disorders of the thyroid gland.

Acknowledgments

The authors wish to express thanks for the aid and supervision given by Dr. J. Doupe and Dr. L. G. Bell. The apparatus used in this study was supplied by the National Research Council of Canada.

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AN EFFECT OF ILL HEALTH ON THE EXCRETION OF 17-KETOSTEROIDS[1]

By William F. Perry

Abstract

The urinary excretion of 17-ketosteroids was measured during the first four hours after waking and during the next 20 hr. of the day in 29 ill adults, 18 males and 11 females, and in 31 control adults, 16 males and 15 females. The ill subjects excreted a smaller fraction of their total daily output in the first four hours of the day than did the controls. This finding is ascribed to defective secretion of 17-ketosteroid precursors by the adrenal cortex.

Introduction

Various workers (6, 10, 14, 15) have demonstrated that the urinary excretion of 17-ketosteroids is usually lowered during sleep. Pincus (8, 9), besides noting decreased excretion during sleep, noted that ketosteroids were excreted at a higher rate during the morning hours than during the remainder of the day He has recently (10) also shown that this diurnal rhythm of excretion is only slightly affected by aging.

Several investigators (3, 4, 6, 13) have noted that ill health is associated with lowered excretion of urinary 17-ketosteroids. Most of these determinations have been carried out on 24-hr. samples of urine or on urine produced during shorter intervals but expressed on a 24-hr. basis. Therefore it was considered of interest to study the effect of ill health not only on the total 24-hr. production of ketosteroids but also on the reported diurnal rhythm in excretion and particularly the effect of ill health on the morning rise in ketosteroid excretion noted by Pincus (8, 9).

Methods

Because there is considerable daily variation in ketosteroid excretion (6, 14), urine was collected from most subjects on at least two separate days in a seven-day period. The mean figures for these days were used in calculating the results.

On waking, the subject discarded the overnight urine, and all urine produced during the next four hours was collected and assayed for its ketosteroid content. The urine produced during the next 20 hr. was collected separately and also assayed. Thus the total 24-hr. production could be obtained. Breakfast was taken early in the four-hour collection period; the other meals were taken during the 20-hr. collection period. The urine was either processed immediately after collection or stored in the frozen state. Urine stored in this manner has been found to undergo no deterioration in ketosteroid content for as long as four months.

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[1] *Manuscript received May 11, 1948.*

Contribution from the Departments of Medical Research of the University of Manitoba and the Winnipeg General Hospital, Winnipeg, Man.

Estimations of the 17-ketosteroid content were made using the hydrolysis and extraction procedure of Miller, Mickelsen, and Keys (7). Colorimetry was performed on the extracts using the modifications suggested by Cahen and Salter (2) to eliminate nonspecific chromogens. By this method, duplicate determinations checked within 5%. Androsterone* was used as a reference standard. Standard statistical methods as described by Fisher (5) and Snedecor (12) were used in analyzing the results.

Subjects

All the hospitalized subjects were patients on the wards of the Winnipeg General Hospital.

A. Controls

(i) Sixteen males of whom eight, ranging in age from 20 to 23 years, were normal people at their usual occupations; the other eight, ranging in age from 35 to 64 years, were hospitalized for various elective operations or investigational procedures, but their general health was not affected. These subjects are listed in Table I.

(ii) Fifteen females aged 16 to 61 years; three of these were working at their usual occupations; the other 12 were hospitalized for investigational procedures similar to those for some of the control males and, like the control males, they did not appear to be ill in a general sense. Their illnesses consisted of two cases each of bunions, minor injuries, psychoneurosis, and nontoxic goiter; one case each of pregnancy, fractured pelvis (one month after injury), appendicitis (one week postoperative), and quiescent rheumatoid arthritis.

In both control groups ketosteroid determinations were done either prior to any operative procedures or at a sufficient time afterward to avoid the elevation of ketosteroid excretion that follows operative procedures and other stress situations (6).

B. *Ill Subjects*

(i) Eighteen males aged 24 to 85 years. These patients and the diagnoses of their illnesses are listed in Table II.

(ii) Eleven females aged 16 to 70 years. In this group the illnesses were: two cases each of myxedema and Grave's disease and one case each of subacute appendicitis, pulmonary edema, hemiplegia, carcinoma of the pancreas, carcinoma of the stomach, carcinoma of the rectum, and Cushing's syndrome.

The subjects in these two groups were designated ill on the basis of clinical criteria. In general they had lowered hemoglobin levels and elevated sedimentation rates in contrast to the control group in which these values were usually normal. The ill subjects showed no evidence of renal failure as judged from blood urea nitrogen values and from the variability in urine concentration.

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* *The Androsterone used in this investigation was kindly donated by the CIBA CO., LTD., Montreal, Que.*

Results

The individual and average 4-, 20-, and 24-hr. excretions of 17-ketosteroids by the 16 control males are shown in Table I. The four-hour excretion is also shown as a percentage of the total 24-hr. excretion. The mean value of these percentages for the control group of 16 males was 22.5%.

TABLE I

Excretion of 17-ketosteroids by 16 control males

Name	Age	Diagnosis	4-Hr. excretion, mgm.			Mean 4-hr. excretion	20-Hr. excretion, mgm.			Mean 20-hr. excretion	Mean 24-hr. excretion	Mean 4-hr. as % 24-hr. excretion
			1	2	3		1	2	3			
G.B.	20	Normal	2.1	3.0	2.5	2.5	11.0	10.7	11.0	10.9	13.4	18.7
M.B.	26	Normal	3.4	3.5	2.1	3.0	11.8	12.4	11.2	11.8	14.8	20.2
R.C.	27	Normal	3.3	4.0	3.1	3.4	11.3	17.9	14.2	14.4	17.8	19.0
J.C.	24	Normal	3.0	3.2	2.4	2.9	11.1	10.4	11.4	10.9	13.8	21.0
W.P.	28	Normal	2.3	3.7	3.8	3.2	9.6	9.8	10.5	9.9	13.2	24.2
C.K.	33	Normal	3.3	4.3	2.9	3.5	7.1	7.8	14.3	9.7	13.2	26.5
R.D.	24	Normal	3.1	3.2	5.2	3.5	12.5	9.0	9.9	10.8	14.3	24.5
L.K.	20	Normal	4.4	4.8	–	4.6	11.1	15.5	—	13.3	17.9	25.6
H.C.	36	Malunited fracture	1.3	–	–	1.3	4.3	—	—	4.3	5.6	23.2
P.L.	53	Auricular fibrillation	2.0	–	–	2.0	7.2	—	—	7.2	9.2	21.7
H.M.	41	Intervertebral disc	2.3	–	–	2.3	7.5	—	—	7.5	9.8	23.5
N.P.	37	Fractured tibia (one month later)	2.8	1.9	1.9	2.2	8.6	9.3	6.2	8.0	10.3	21.4
C.S.	62	Medial meniscus	2.6	2.2	–	2.4	4.7	5.8	—	5.2	7.6	31.5
T.S.	35	Intervertebral disc	2.5	–	–	2.5	10.2	—	—	10.2	12.7	19.7
H.W.	64	Bronchial asthma	1.4	1.2	0.7	1.1	4.9	2.4	3.9	3.7	4.8	22.9
N.G.	31	Nontoxic goiter	1.9	2.0	–	1.9	4.7	10.4	—	7.6	9.5	20.0
Mean values						2.2				9.1	11.7	22.5

In Table II are presented the individual and average 4-, 20-, and 24-hr. excretions of the 18 sick males, each four-hour excretion being again shown as a percentage of the total 24-hr. excretion. In four hours these 18 ill males excreted on the average only 14.7% of their 24-hr. output.

In Table III is presented a summary of the data obtained on these two groups and of analogous data obtained on the two groups of females. It will be seen that the control females had an average four-hour excretion of 19.3% of their total 24-hr. output, while the sick females averaged only 14.5%.

The ill subjects excreted smaller absolute amounts of ketosteroids than the well. This is in agreement with previous findings (3, 4, 6, 13). Because there was considerable variation in the fraction excreted in the first four hours

TABLE II

EXCRETION OF 17-KETOSTEROIDS BY 18 ILL MALES

Name	Age	Diagnosis	4-Hr. excretion, mgm.			Mean 4-hr. excretion	20-Hr. excretion, mgm.			Mean 20-hr. excretion	Mean 24-hr. excretion	Mean 4-hr. as % 24-hr. excretion
			1	2	3		1	2	3			
I.I.	71	Prostatic hypertrophy	0.45	0.58	0.56	0.53	4.6	3.7	4.2	4.3	4.8	11.0
W.J.	61	Prostatic carcinoma	0.54	0.55	—	0.55	4.2	5.7	–	4.4	4.9	11.2
W.L.	67	Prostatic carcinoma	1.10	1.40	0.63	0.95	5.4	4.8	4.4	5.1	6.0	15.8
H.McI.	69	Prostatic hypertrophy	2.60	—	—	2.60	5.5	–	–	5.5	8.1	32.0
W.McN.	50	Prostatic carcinoma	1.10	1.40	—	1.30	6.4	6.8	–	6.4	8.0	16.2
W.F.	35	Pelvic fracture	0.47	1.80	0.39	0.88	5.5	2.6	5.6	4.9	5.8	15.2
R.McR.	77	Prostatic hypertrophy	1.00	0.70	1.00	0.90	7.9	7.6	5.7	7.0	7.9	11.4
L.S.	64	Cirrhosis	0.34	—	—	0.34	5.1	–	–	5.1	5.4	6.4
P.M.	35	Addison's disease	0.62	—	—	0.62	4.0	–	–	4.0	4.6	13.4
R.B.	24	Hepatitis	0.88	—	—	0.88	6.0	–	–	6.0	6.9	12.8
W.H.	71	Prostatic hypertrophy	0.95	—	—	0.95	7.0	–	–	7.0	7.9	12.0
J.P.	85	Prostatic carcinoma	0.50	0.60	0.60	0.56	3.0	3.9	3.7	3.5	4.1	13.7
H.W.	72	Prostatic hypertrophy	0.84	1.20	0.21	0.75	3.5	1.7	1.8	2.3	3.0	25.0
J.W.	74	Prostatic carcinoma	0.27	0.96	—	0.61	3.7	2.9	–	3.3	4.0	15.2
S.L.	73	Myxedema	0.32	0.57	0.36	0.32	3.4	4.7	3.0	3.7	4.1	7.8
S.S.	75	Prostatic hypertrophy	0.56	1.20	0.77 1.20	0.93	4.7	6.7	5.6 5.2	5.5	6.5	14.3
H.T.	72	Prostatic hypertrophy	0.74	0.79	0.82	0.79	5.3	6.9	5.2	5.8	6.6	12.0
H.B.	66	Malnutrition	0.40	—	—	0.40	4.1	–	–	4.1	4.5	8.9
Mean values						0.83				4.9	5.7	14.7

TABLE III

EXCRETION OF KETOSTEROIDS BY CONTROL AND ILL SUBJECTS

Group	Mean 4-hr. excretion, mgm.	Mean 20-hr. excretion, mgm.	Mean 24-hr. excretion	Mean 4-hr. excretion % of 24-hr. excretion	Standard deviation	Range
Control males (16)	2.20	9.1	11.7	22.5	3.3	18.7 - 31.5
Sick males (18)	0.83	4.9	5.7	14.7	6.0	7.8 - 32.0
Control females (15)	1.39	5.8	7.2	19.3	4.0	11.4 - 25.7
Sick females (11)	0.83	5.2	6.0	14.5	3.4	10.2 - 21.6

in all groups, an analysis of variance was performed to test the significance of the difference in four-hour percentage excretions of the four groups. In Table IV are set forth the calculated probabilities of the differences in the four-hour percentage excretion being due to chance.

Thus there is a highly significant difference between the four-hour percentage excretions of the sick males (14.7%) and of the control males (22.5%) and also between the sick females (14.5%) and the control females (19.3%).

TABLE IV

SIGNIFICANCE OF DIFFERENCE BETWEEN GROUPS

Group	*P*
Control males vs. sick males	$<.01$
Control females vs. sick females	$<.01$
Control females vs. control males	$>.01$ but $<.05$
Sick males vs. sick females	$>.05+$

It would appear in addition that control males excrete a significantly higher fraction of their total 24-hr. production of ketosteroids in the first four hours of the day than do control females.

Discussion

Before discussing the interpretation of the results it is necessary to consider factors involved other than illness. The most apparent of these is hospitalization. That this does not explain the difference between the groups is shown in Table I. It will be seen that the normal active individuals had the same four-hour percentage excretion as the hospitalized subjects. This suggests that hospitalization per se does not substantially alter the fraction of the total ketosteroid output excreted in the first four hours. Defective kidney function could not be held to be a responsible factor.

The results reported above show that ill health, in addition to its generally accepted role in decreasing the absolute amounts of ketosteroids excreted, also tends to abolish the morning increase in excretion that is characteristic of the healthy state. The normal morning rise in excretion has been interpreted by Pincus (8, 9) as a reaction to the stress of daily activities that begin on waking. Forbes *et al.* (6) have shown that chronically ill hospital patients, unlike normal persons, are unable to respond to various stress situations by excreting greater amounts of ketosteroids. They ascribe this phenomenon in the healthy to a release of cortical "N" hormone from the adrenal cortex. The chronically ill do not respond in this manner and are considered to be in the resistant phase of the "Adaptation Syndrome" of Selye (11).

The altered diurnal rhythm of ketosteroid excretion in the group of ill subjects may therefore be due to lack of adrenal cortical response to the stress of the numerous, diverse activities that begin on waking. If so, the estimation of this fraction may be of some value in assessing the functional state of the androgenic or "N" part of the adrenal cortical system.

The moderately significant difference in the four-hour fractions of the control males and females suggests possibly a greater reactivity on the part of the male. However, this difference does not appear large and its cause has not been investigated. Possibly, ketosteroids of testicular origin are also subject to variations in excretion as are those of cortical origin.

Acknowledgments

The work reported in this paper was undertaken with the aid of a grant from the Division of Medical Research, National Research Council, Canada.

The author is indebted to Dr. Joseph Doupe and Prof. I. M. Thompson for advice and criticism and to Miss Marion Sugden for technical assistance.

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PRESERVATION OF ACETYLCHOLINE IN HUMAN CEREBROSPINAL FLUID: A SIMPLE METHOD FOR CLINICAL USE[1]

BY DONALD B. TOWER

Abstract

A simple and effective method for preservation of acetylcholine in human cerebrospinal fluid has been described. The method depends on the use of acidification and prostigminization of cerebrospinal fluid. No detectable loss of acetylcholine for periods up to seven days or longer has been observed. The method has been satisfactorily used in clinical investigations on more than 200 cerebrospinal fluid samples from over 100 neurological and neurosurgical patients.

Introduction

One of the principal difficulties in the study of the acetylcholine–cholinesterase system is the instability of the acetylcholine molecule. A satisfactory method for its preservation until assays are carried out is essential in biological work. Babkin *et al.* (1) have reported a method for the preservation of acetylcholine in whole blood for periods up to seven days. The method depends on the inhibition of cholinesterase activity by the combined use of prostigmine, acidification, and refrigeration. Considerable variability in results was encountered because of the difficulty of pH control in the presence of the large electrolyte and protein buffer of the blood and because of the large quantity of cholinesterases free to act before preservation could be achieved (3).

It has been possible to work out a successful modification of this method for cerebrospinal fluid because of three fundamental differences between cerebrospinal fluid and blood. *First*, the buffering power of cerebrospinal fluid is very small, so that acidification and maintenance of an acid pH present no problem. *Second*, the cholinesterase activity of cerebrospinal fluid is only about 0.5% of that of whole blood (5). And *third*, the observed substrate concentrations of 0.02 to 10 μgm.%* acetylcholine have proved to be suboptimal, so that enzyme activity is markedly impaired (5). Thus, the loss of acetylcholine before preservation is negligible.

Procedure

The method of acetylcholine preservation was developed for the assay procedure employing the isolated ventricle of the mollusc, *Venus mercenaria* (4), but it may be adapted to any of the other standard methods of assay.

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[1] *Manuscript received October 6, 1948.*

Contribution from the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal, Canada.

This study was supported by a grant from the Rockefeller Foundation.

* *μgm.% = micrograms per 100 ml.*

(1) Freshly drawn cerebrospinal fluid (5.0 ml.) is pipetted into a clean test tube, containing 1.0 ml. of 1 : 4400 prostigmine (neostigmine) methyl sulphate, 1.0 ml. of 0.1 *N* hydrochloric acid, and 2.0 ml. of 0.9% sodium chloride. (The pH of the preserved solutions varies between 5.5 and 6.0, as measured with a Beckman pH meter.)

(2) After the contents are thoroughly mixed, the solution is refrigerated at about 5° C. until ready for use.

(3) Immediately prior to testing the solution, 1.0 ml. of 0.05 *N* sodium hydroxide is added to partially neutralize the acid (raising the pH to 6.9–7.3). Final volume of the preserved solution is 10.0 ml., which represents a 1 : 2 dilution of the original cerebrospinal fluid sample.

Cerebrospinal fluid samples preserved in this manner show no detectable loss of acetylcholine for periods of seven days or longer. The preservation formula is summarized in Table I.

TABLE I

FORMULA FOR PRESERVATION OF ACETYLCHOLINE IN HUMAN CEREBROSPINAL FLUID

Cerebrospinal fluid	5.0 ml.
Prostigmine, 1 : 4400	1.0 "
Hydrochloric acid, 0.1 *N*	1.0 "
Sodium chloride*, 0.9%	2.0 "
	9.0 ml. (pH = 5.5 to 6.0)
Sodium hydroxide, 0.05 *N* (to neutralize)	1.0 ml.
Final volume	10.0 ml. (pH = 6.9 to 7.3)

* *Optional.*

The 10.0 ml. volume was found most convenient to provide sufficient quantity for multiple tests of active and hydrolyzed samples.*

Results

More than 200 samples of human cerebrospinal fluid have been satisfactorily preserved by the method outlined above. As an example of its efficacy over a seven day period, one experimental run (PR-8) is illustrated. The make-up of the samples is tabulated in Table II.

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* *The 2.0 ml. of 0.9% sodium chloride in the formula may be omitted, since it has been included only to give a 1 : 2 dilution. In calculating the concentrations of acetylcholine in the original cerebrospinal fluid sample, the volume of the assay chamber, the preservation dilution factor, and the amount of acetylcholine measured by assay are multiplied together. Thus, in the results reported here, where a 20.0 ml. assay chamber was used, the original cerebrospinal fluid samples contained 2 × 20 or 40 times the amounts of acetylcholine recorded at assay.*

TABLE II

EXPERIMENTAL PRESERVATION OF ACETYLCHOLINE IN HUMAN CEREBROSPINAL FLUID

(EXPT. NO. PR-8)

MAKE-UP OF SAMPLES

Constituents	Concentration	Samples (vol. in ml.)						
		M-1	M-2	N-1	N-2	O	P-1	P-2
Prostigmine	1:4400	1.0	1.0	—	—	1.0	1.0	1.0
Hydrochloric acid	0.1*N*	1.0	1.0	—	—	1.0	1.0	1.0
Sodium chloride	0.9%	—	—	3.0	3.0	2.0	5.0	5.0
Pooled human CSF	—	5.0	5.0	5.0	5.0	5.0	—	—
Acetylcholine	10.0 μgm.%	2.0	—	2.0	—	—	2.0	—
Acetylcholine	5.0 μgm.%	—	2.0	—	2.0	—	—	2.0
Sodium hydroxide*	0.05*N*	1.0	1.0	—	—	—	—	—
Sodium hydroxide*	0.1*N*	—	—	—	—	1.0	1.0	1.0
Total volume		10.0	10.0	10.0	10.0	10.0	10.0	10.0
Calculated final concentration of acetylcholine in μgm.% (for 1.0 ml. sample in 20.0 ml. chamber)		0.1	0.05	0.1	0.05	0.0	0.1	0.05

* *Added immediately before testing.*

A pool of four human cerebrospinal fluids, previously shown to contain no acetylcholine, was used. A series of identical samples for testing on successive days was set up as indicated (Table 11).

The results of assay after 24 hr. of refrigeration are shown in Fig. 2. It can be seen that unpreserved samples, N-1 and N-2, showed little or no acetylcholine remaining after 24 hr. They gave a *Venus* heart tracing similar to that of Sample O, which contained no acetylcholine. In contrast, Samples M-1, M-2, P-1, and P-2 produced the same percentage of inhibition of the *Venus* heart beat as fresh aqueous known solutions of the same concentration. This is graphically demonstrated in Fig. 1, where the measured percentage inhibition of the *Venus* heart beat (in Fig. 2) has been plotted against the acetylcholine concentration in μgm.%. The correspondence of the points for fresh aqueous known solutions and for preserved M and P solutions of the same concentration shows no detectable loss of acetylcholine to have occurred in 24 hr.

In Fig. 3 a portion of the *Venus* heart tracing after seven days, preservation shows that in preserved samples M-1 and M-2 no detectable loss of acetylcholine occurred, while in unpreserved samples N-1 and N-2 no detectable acetylcholine remained. Several similar experiments were run, all of which showed satisfactory preservation of known amounts of acetylcholine in human cerebrospinal fluids for periods up to 10 days.

In practice unknown cerebrospinal fluid samples from ward patients have all been tested within seven days of the time of withdrawal and preservation of the sample. *Venus* heart tracings on two such cases of epilepsy with positive cerebrospinal fluid acetylcholine assays are given in Fig. 4. The results of cerebrospinal fluid studies on 130 neurological and neurosurgical patients and on experimental animals, using this method of acetylcholine preservation, will be reported elsewhere (2, 5, 6, 7).

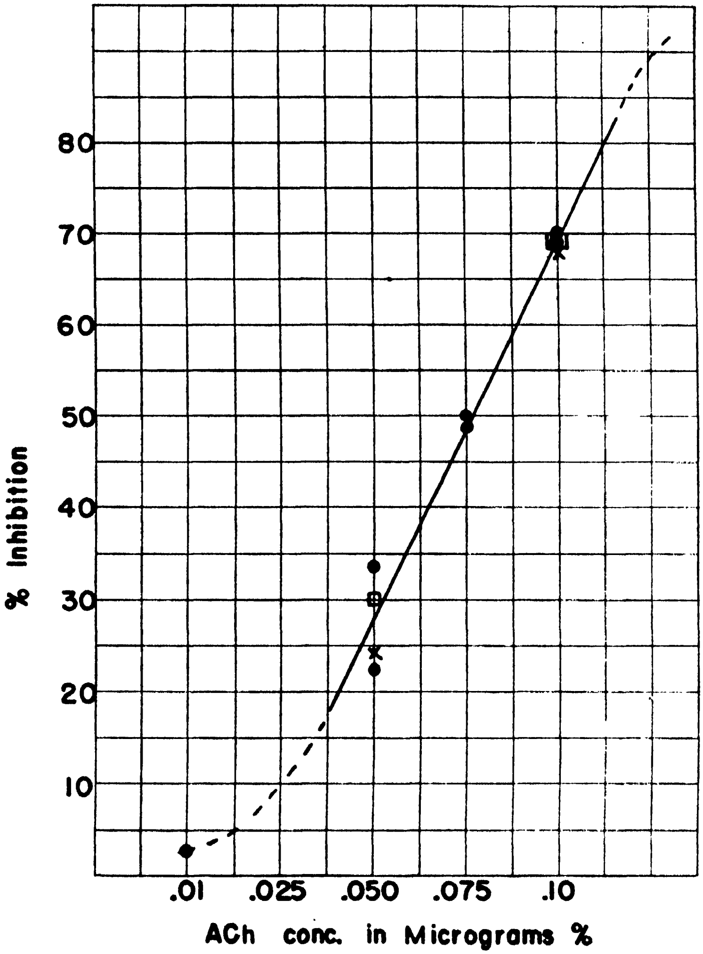

FIG. 1. *Acetylcholine concentrations plotted against percentage inhibition of assay test object for known fresh aqueous solutions and preserved solutions, derived from Fig. 2. M-5161.* ● *= Fresh solutions.* □ *= P (preserved solutions).* × *= M (preserved solutions).*

Acknowledgments

The author wishes to express his appreciation for the advice of Dr. Donald McEachern and the assistance of Mr. Murray Bornstein in carrying out these experiments.

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Explanation of Figures

PLATE I

FIG. 2. *Acetylcholine assays on human cerebrospinal fluid samples preserved for 24 hr. (Expt. PR-8).*

ACh = Acetylcholine in μgm.%
w = Wash
M, N, O, P = Preserved and unpreserved samples (See Table II)
h = Hydrolyzed samples.

PLATE II

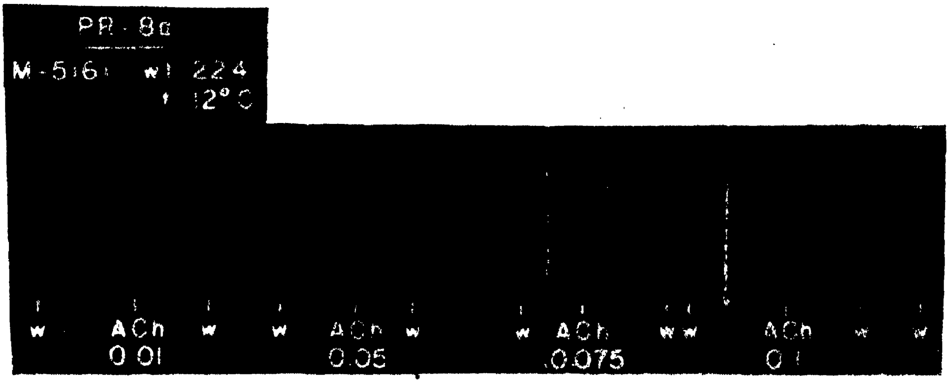
FIG. 3. *Acetylcholine assays on human cerebrospinal fluid samples preserved for seven days (Expt. PR-8).*

ACh = Acetylcholine in μgm.%
w = Wash
M, N, O = Preserved and unpreserved solutions (See Table II)
h = Hydrolyzed samples.

FIG. 4. *Acetylcholine assays on cerebrospinal fluids of human epileptic patients; samples preserved by the author's method.*

ACh = Acetylcholine in μgm.% (Note: 10^{-10} = 0.01 μgm.%)
w = Wash
h or hyd = Hydrolyzed samples
1217GN = Patient with focal left temporal lobe seizures, cause unknown
916RMacD = Patient with focal right temporal lobe seizures due to calcified ependymoma

PLATE I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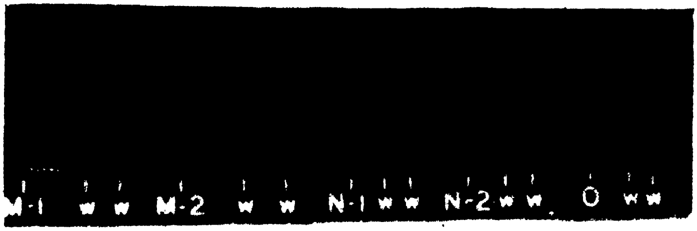
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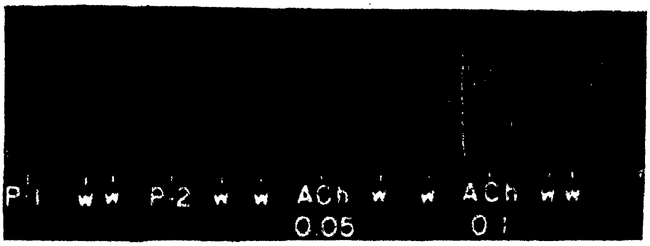
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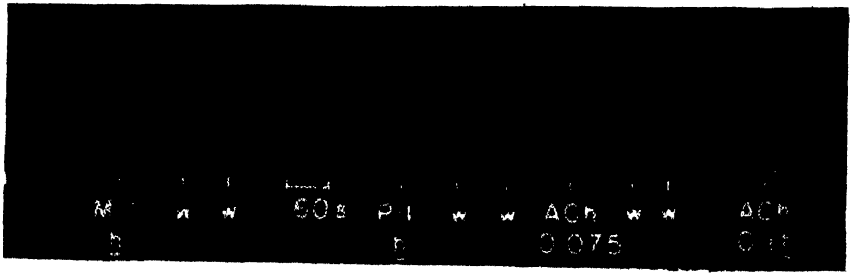
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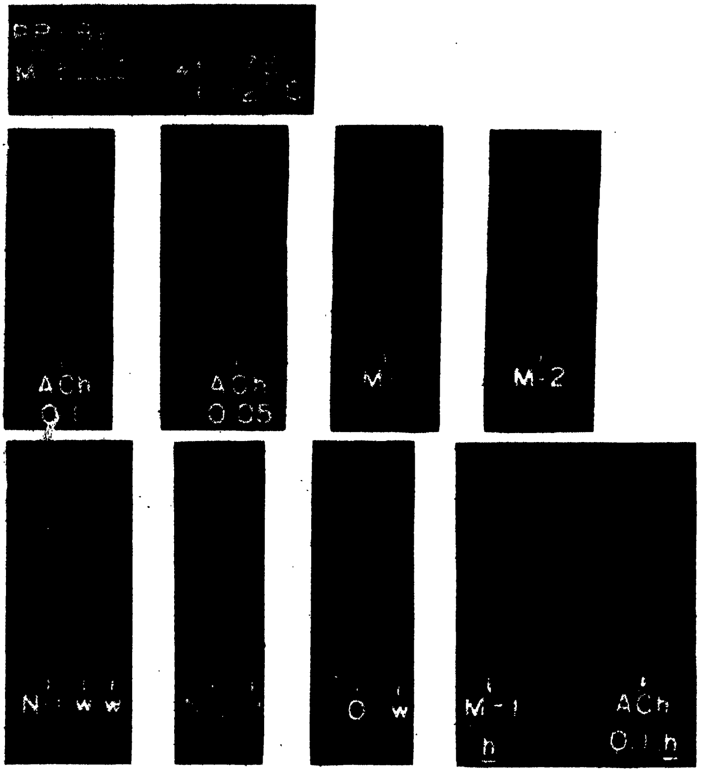
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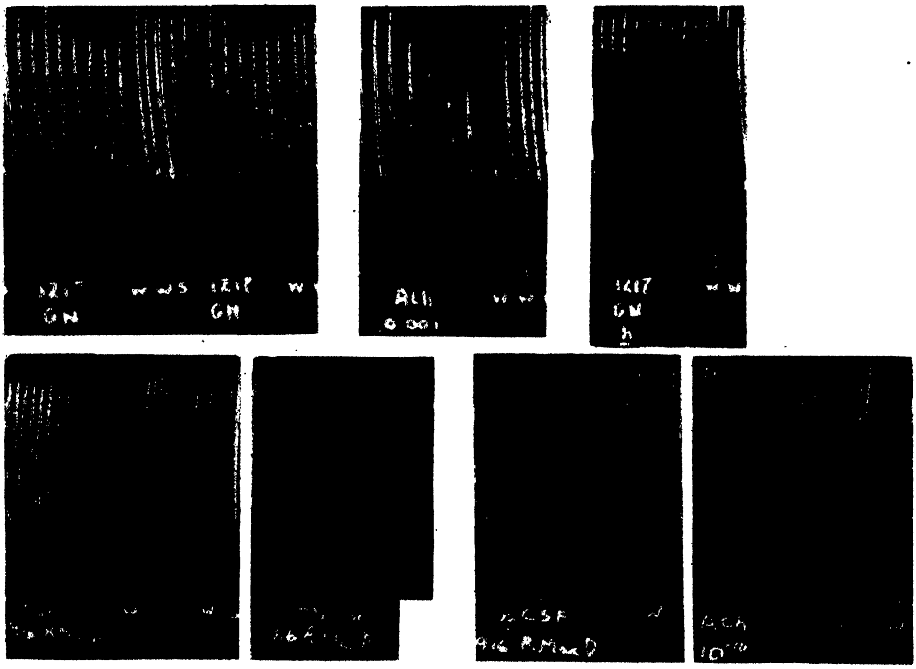
3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w w S
1218
GM
w
ACh
0.001
w w
1417
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THE RELATIONSHIP BETWEEN SERUM ALKALINE PHOSPHATASE AND DIETARY ESSENTIAL AMINO ACIDS[1]

BY JULES TUBA, MAX M. CANTOR, AND A. GERALD RICHARDS

Abstract

It has been found that, when weanling rats are placed on a diet containing low protein barley (protein = 8.5%) or on diets containing low protein barley with supplements of casein or individual essential amino acids, with the exception of lysine and methionine, the level of serum alkaline phosphatase is related in an inverse fashion to the rate of growth. The addition to the basal low protein diet of methionine or casein, i.e., of labile methyl groups, results in the lowering of serum alkaline phosphatase activity towards normal levels. This is considered to reflect a return to normal of fat mobilization.

Introduction

It was reported by Hough *et al.* (4, 5) that dogs fed a high fat, protein deficient diet showed a pronounced elevation of serum alkaline phosphatase values and that these levels decreased following the return of the animals to a diet containing adequate protein. They presented evidence that the lipotropic substances, choline and methionine, reverse the effect of protein deficiency on serum phosphatase. It is apparent from their work that fat metabolism is related to serum phosphatase activity and that the phosphatase-decreasing action of proteins is associated with the methionine content of the diet. A direct correlation between the activity of serum alkaline phosphatase and the amount of fat ingested has been shown clearly by Cantor *et al.* (1). They showed that a decrease below full daily requirements in the food consumption of adult male rats produced a fall in serum alkaline phosphatase proportional to the amount of fat consumed daily rather than to the lowered calorie intake.

The work reported here is a preliminary study designed to determine the effect on serum alkaline phosphatase levels in growing rats of: (1) a low protein diet, (2) a low protein diet supplemented with each of eight essential amino acids.

Experimental

Weanling male albino rats, 21 days old, were used for the feeding experiments. They were housed individually in metal cages and given tap water to drink. They were fed daily at 9 a.m. except on days when blood samples were taken and then food was provided immediately after bleeding. There were at least six animals in each of the groups used for the various diets.

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[1] *Manuscript received August 9, 1948.*

Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council, Ottawa, Canada.

Tail blood was collected by "milking" about 0.5 ml. of blood into microcentrifuge tubes for obtaining serum. Within an hour of collection, serum was removed and stored in a refrigerator. Enzyme activity was invariably estimated within two or three days. Serum phosphorus and alkaline phosphatase* were determined by the Gould and Schwachman modification (3) of the Shinowara micro-method (8). Weights of individual animals in each group were recorded twice a week during the feeding period, which was generally about six weeks, and gain or loss in weight was noted. These changes were averaged for each group.

The basal diet was finely ground low protein barley (L.P.B.) containing 9.1% protein and 3% fat, supplemented with fat, salt mixture, and vitamins in the following proportions:

L.P.B.	930 gm.
McCollum's salt mixture	40 gm.
Crisco	30 gm.
Cod liver oil	20 gm.

To this was added 10 mgm. thiamine hydrochloride, 10 mgm. pyridoxine hydrochloride, 20 mgm. calcium pantothenate, 10 mgm. niacin, and 1 gm. choline chloride, all dissolved in 15 ml. of 95% alcohol; and 20 mgm. riboflavin distributed in 5 gm. of glucose. All the ingredients were mixed intimately and passed through a fine wire sieve three times. This basal diet, containing 8.5% protein, was stored in tightly stoppered dark bottles.

For the supplemented diets the following amounts of individual amino acids and of casein were contained in 1 kgm. of each diet:

Lysine monohydrochloride	7.80 gm.
Leucine	2.30 gm.
dl-Methionine	8.80 gm.
dl-Phenylalanine	5.20 gm.
dl-Threonine	5.20 gm.
Tryptophane	1.86 gm.
Valine	1.86 gm.
Histidine monohydrochloride	3.00 gm.
Casein (SMACO vitamin-free)	80.00 gm.

The barley and an estimate of its essential amino acid content were kindly supplied to us by the Department of Animal Science, University of Alberta. Each amino acid was added to the basal diet in an amount sufficient to raise the level of the acid present in L.P.B. and supplement to the requirement set by Rose (6) on the basis that food consumption was 10 gm. per animal per day. The addition of 8% casein to the basal diet raised the protein content to 16.5% and increased the daily intake of essential amino acids to approximately that required by Rose. Where the racemic form of the amino acid was used, the calculated amount was doubled. Arginine was not added to the basal diet since the barley contained a large excess of this acid. A group of animals fed the stock laboratory diet of Purina Fox Checkers containing not less than 20% protein served as a control. All diets were fed for a period of 40 days.

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* *The unit of phosphatase activity is equivalent to 1 mgm. of phosphorus liberated as phosphate ion during one hour of incubation at 37° C. with a substrate containing sodium β-glycerophosphate at pH for the alkaline enzyme of 9.3.*

The Effect of L.P.B. and Supplemented L.P.B. on the Rate of Growth of Normal Weanling Rats

Data on these experiments is presented in Table I in terms of average weight gained per group at 10 day intervals.

TABLE I

The relationship between diet and weight gain

Diets	No. of animals	Weight gain in grams			
		Days on diet			
		10	20	30	40
Checkers	10	28	78	125	165
L.P.B.	20	10	22	36	53
L.P.B. and casein	10	31	75	102	120
L.P.B. and lysine	10	12	26	42	57
L.P.B. and leucine	6	12	29	40	46
L.P.B. and methionine	6	14	25	36	50
L.P.B. and threonine	6	10	22	35	48
L.P.B. and valine	6	15	32	47	62
L.P.B. and histidine	6	12	27	42	62
L.P.B. and phenylalanine	6	7	16	27	40
L.P.B. and tryptophane	6	6	15	26	40

Growth of weanling rats on L.P.B. is greatly retarded. The addition of casein to L.P.B. provides partial protein replacement but the supplementation of L.P.B. with individual amino acids produces no marked improvement in growth and, in some instances, notably with phenylalanine and tryptophane, there seems to be further retardation of growth.

The Effect of L.P.B. and Supplements on Serum Alkaline Phosphatase Activity in Normal Rats

Serum alkaline phosphatase was estimated at the commencement of feeding and at 3.0, 4.5, and 6 weeks. The results presented in Table II are for the same animals as those referred to in Table I and are the averages of groups.

TABLE II

Serum alkaline phosphatase levels in Shinowara units of growing rats on low protein diet and supplemented low protein diets

Diets	Serum alkaline phosphatase units/100 ml.		
	3 Weeks	4.5 Weeks	6 Weeks*
Checkers (20% protein approx.)	108	132	104 ± 28
L.P.B. (8.5% protein)	186	187	179 ± 38
L.P.B. and casein (16.5% protein)	183	148	160 ± 25
L.P.B. and methionine	196	138	139 ± 13
L.P.B. and lysine	241	253	234 ± 49
L.P.B. and phenylalanine	192	214	212 ± 25
L.P.B. and tryptophane	256	243	203 ± 33
L.P.B. and histidine	251	215	191 ± 18
L.P.B. and leucine	196	220	169 ± 16
L.P.B. and threonine	201	168	164 ± 26
L.P.B. and valine	237	235	178 ± 31

* *Standard deviations are indicated for phosphatase values at end of experiment.*

It will be seen that at the termination of the experiment animals fed the L.P.B. diet showed an elevation of about 75% in serum alkaline phosphatase activity as compared with the normal animals given the stock laboratory diet. Supplementing the L.P.B. with casein reduced alkaline phosphatase activity slightly below the values for the L.P.B. group. There is a marked decrease in enzyme activity with methionine. A further increase in phosphatase activity above that noted in the L.P.B. group is present in animals receiving supplements of lysine, phenylalanine, and tryptophane. Animals receiving the other supplements showed no marked change from those in the L.P.B. group.

Effect of Changing Animals from L.P.B. Plus Lysine Diet to Stock Diet

Table III presents the changes in phosphatase activity and rate of weight gain in 10 animals. These animals were kept on the L.P.B. and lysine diet for 40 days and then returned to the stock laboratory diet for eight days.

TABLE III

Effect of normal diet on animals previously fed L.P.B. and lysine

	L.P.B. + lysine diet			Stock diet
	21 Days	30 Days	40 Days	48 Days
Alkaline phosphatase units per 100 ml.	241	253	234	131
Weight gain in grams	12	26	57	98

It appears from Table III that the increase in alkaline phosphatase is related to the degree of retardation in growth. After nearly six weeks on L.P.B. and lysine, phosphatase activity was 234 units per 100 ml. of serum while the weight gain was only 57 gm. In the eight day period following the feeding of the stock laboratory diet, the phosphatase activity was reduced to an almost normal level and there was a weight gain of 41 gm.

Table IV presents a compilation of the changes in phosphatase activity and growth at the end of 40 days of the test period on the various diets used.

TABLE IV

THE RELATIONSHIP BETWEEN GROWTH RATE ON VARIOUS DIETS AND SERUM ALKALINE PHOSPHATASE ACTIVITY

Diet	Weight gain in 40 days	Alkaline phosphatase units/100 ml.
Stock diet	165	104
L.P.B. and casein	120	160
L.P.B. and valine	69	178
L.P.B. and histidine	62	191
L.P.B. and lysine	57	234
L.P.B.	53	180
L.P.B. and methionine	50	139
L.P.B. and threonine	48	164
L.P.B. and leucine	46	169
L.P.B. and phenylalanine	40	212
L.P.B. and tryptophane	40	203

It will be seen that groups fed L.P.B. and phenylalanine or L.P.B. and tryptophane showed the least weight gain and the highest phosphatase levels apart from the group on L.P.B. and lysine. In general, animals in other groups showed a similar correlation, and the less the weight gain, the higher the phosphatase level with the exception of the groups maintained on L.P.B. and methionine or lysine supplements.

The behavior of lysine in departing from the general trend cannot be explained. It should be noted, however, that this amino acid has been shown to be important for the synthesis of proteins in the liver by Gillespie *et al.* (2), and it is possible that alkaline phosphatase functions in such processes.

The effect of methionine used to supplement a diet low in protein is very definite and this observation is in conformity with the findings of Hough and his co-workers. Hough fed to dogs a diet containing 33% fat, a level much higher than 8% fat received by the rats used in the work reported here. Nevertheless, it seems that in both instances the increased phosphatase values represent a response to interference in the mobilization of fat, and that the addition of labile methyl groups either in the form of methionine or protein favorably affects both these activities. It should be noted, also, that Salmon (7) has shown with rats that in diets containing 18% or less casein the primary deficiency, resulting in impaired growth, is labile methyl groups, unless supplements of methionine or choline are added. Our work, in so far as it has gone, is in accord with Salmon's investigations. Further experiments are now in progress to clarify the various relationships between serum alkaline phosphatase, dietary protein, and certain amino acids.

Acknowledgments

The authors wish to express thanks to John Wyeth and Brother (Canada) Limited, for the vitamins and vitamin-free casein used in this investigation.

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THE PREPARATION OF ISINGLASS SOLUTIONS SUITABLE FOR TRANSFUSION PURPOSES[1]

By H. D. Bett

Abstract

The possibilities of producing isinglass solution in sufficiently large quantity to permit adequate clinical trial on a uniform batch have been investigated. A satisfactory process has been developed using fresh frozen hake sounds. Technical details of this preparation are presented in this paper, together with comments on the occurrence and prevention of pyrogens in transfusion fluids. The formation of pyrogens was a continual source of trouble until sterile technique for handling large volumes of material was developed. Technical details are presented, including a method for the removal of pyrogenic substances when, in spite of precautions, these are present. Preparations free from pyrogenic substances can be produced through the use of aseptic technique or by filtration through sterilizing filter pads. In using the latter method it was necessary to limit the quantity of liquid that was filtered through any single pad.

Introduction

During the course of World War II many substances were considered for use as blood substitutes. Gelatin received considerable attention in this respect. Jorns (5) indicated that colloids in isotonic sodium chloride solutions were useful as blood substitutes. Kleinberg and his associates (6) found gelatin effective in the treatment of shock due to trauma. Gordon and associates (2) successfully used gelatin solution to produce recovery in arterial pressure in experimental haemorrhage. Parkins and Lockwood (8) determined that normal dogs tolerated large infusions of gelatin without serious toxic reaction. Gelatin solution was safely administered to normal humans and those in shock by Jacobson and Smyth (4). More recently, Hamilton and associates (3) studied 10 infusion fluids for their effect on haemorrhage in animals. The oxypolygelatin solution reported was superior to physiological saline but less suitable than plasma or 5% albumin solution. McCarthy and Parkins (7) tested the effect of 10 infusion fluids in scald burns in dogs. These authors concluded that the effectiveness of the solutions that afforded the highest effective survival rate could be attributed to sodium salts and water content.

Reports concerning the effectiveness of isinglass solutions as transfusion solutions or blood substitutes are not extensive. Taylor and Waters (11) reported a preparation of isinglass to be useful as a transfusion fluid in experimental haemorrhage in dogs. A further report on this substance was given by Taylor and Moorhouse (10). Pugsley and Farquharson (9) described the use of isinglass solutions in 61 patients. These authors reported that, when properly prepared, isinglass solution appeared to be a safe blood substitute. In a small series of cases of shock the intravenous administration

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[1] *Manuscript received April 7, 1948.*
Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont.

of isinglass solution appeared to be of value. The preparation of isinglass from the dried swim bladder of the hake, by the method of Prof. N. B. Taylor is described by Beveridge and Lucas (1). The amino acid content of isinglass is given in the same paper.

In the method used by Taylor, after the development of the isinglass with calcium hydroxide, large volumes of acetone are required. From experience in our laboratory it was found that 25 liters of acetone were required to produce sufficient isinglass for 500 cc. of a 6% solution such as is proposed for treatment. This makes the preparation of reasonably large amounts a matter of some difficulty. It was also found that contamination of the product with pyrogenic substances was readily encountered when dealing with large quantities of hake sounds in the dried state. Since isinglass appeared to have value as a transfusion fluid, a method was sought to lessen these difficulties. Arrangements were made to have sounds promptly frozen on removal from the fish, and shipped under refrigeration conditions. These sounds* arrived in excellent condition and remained so as long as they were kept frozen. Using frozen sounds as starting material, the following method was developed for the production of a large amount of isinglass solution suitable for clinical trial.

Method

In order to reduce the possibility of pyrogen formation to a minimum after the isinglass had been developed, all of the processing equipment was sterilized.

Forty pounds of fresh frozen hake sounds were placed in running tap water in a stainless steel container until the sounds were no longer hard. This required usually about two hours.

The water was drained off and replaced by 30 gal. of saturated calcium hydroxide solution and 50 lb. of ice. The presence of ice assisted in preventing the physical breakup of the sounds and therefore subsequent losses in handling. The sounds were left in this solution for 24 hr. The solution was then replaced with freshly prepared, saturated calcium hydroxide solution and ice. This process was repeated until the sounds had been subjected to six washings. The material was, of course, kept in a refrigerator throughout this period.

The sounds were then washed in tap water and allowed to stand in water for 24 hr. The water was then removed and replaced by 30 gal. of 0.05 N hydrochloric acid solution. Two such washes were given at an interval of one hour. Ice was present in all these washes as in the case of the calcium hydroxide treatment. Following this, two washes were made with hydrochloric acid of the same concentration but prepared from freshly distilled, pyrogen-free water. No ice was present. (If freshly distilled water cannot be obtained in sufficient quantity, water should be distilled and autoclaved immediately until enough is collected. This procedure usually provides pyrogen-free water.)

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* *The sounds were procured through the co-operation of Mr. E. B. Richardson of the Maritime National Fish Division of Sea Products Limited, Digby, N.S.*

Upon draining the last hydrochloric acid wash from the sounds they were washed in 30 gal. of 1% sodium chloride solution prepared with freshly distilled, pyrogen-free water. This solution was then removed by draining, leaving a gelatinous mass.

In subsequent parts of the process it is particularly important to avoid contamination. The gelatinous mass was transferred to a clean kettle that had been washed by boiling distilled water in it for at least one hour.

The contents were next heated to 90° C., with constant agitation. This agitation served to stir the mixture and to break up the sounds. On reaching this temperature the mixture was usually homogeneous. Eight pounds of Hy-flo* filter aid were added. The temperature was then lowered to 70° C. A small test sample was removed and filtered through paper** on a Buchner funnel. The sodium chloride content was determined by Volhard's thiocyanate procedure, the pH adjusted with 5 *N* sodium hydroxide solution to 7.2 and total solids determined by evaporation of 5 cc. samples in tared dishes. These three operations should be done in less than one hour. The volume before and after filtering having been noted and the volume of the mixture in the kettle being known, calculations could then be made from the three determinations to determine a dilution sufficient to give final values of about 7% total solids, 0.85% in sodium chloride and pH 7.2. Any diluting was done with freshly distilled water or water that had been freshly distilled and autoclaved. The whole was then filtered through a previously steamed plate-and-frame press (six 1 in. frames, 17 in. square, 1 in. thick required). Number 202 Whatman papers backed by cloth were used.

The filtrate was collected in sterile 10-liter bottles and as soon as possible placed in the autoclave. The temperature was brought up to 120° C. (15 lb. pressure) and held for 45 min. When the steam was shut off the autoclave was allowed to reach 100° C. and normal pressure before being opened. The isinglass solution usually contained a precipitate and had to be filtered before final filling.

The mixture was transferred to a sterile pressure tank and the whole filtered under aseptic conditions at 70° C. through St. No. 3*** filter pad by air pressure. This filtrate was then filled into 400 cc. bottles fitted with plastic screw caps and rubber liners. The bottles were filled to within one inch of the top and the screw caps fitted on and tightened. These bottles were then autoclaved, while sealed, for 30 min. This procedure has, to date, always provided material that has been sterile.

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* *Hy-flo procured from Canadian Johns-Manville Company, Toronto.*

** *Whatman 202 was found satisfactory.*

*** *St. No. 3 from Hercules Filter Corporation supplied by General Filtration and Engineering Limited, of Toronto.*

Approximately 60 bottles (400 cc. per bottle) may be prepared from 40 lb. of sounds.

Pyrogens

It has been found possible to prevent the formation of pyrogens by starting with fresh frozen sounds and by keeping exposure to contaminants at a minimum at all times. The sterilization of all material with which the isinglass solution may come in contact, such as rubber hose, extraction kettles, filter papers, and cloths has been found to be essential. In the event that pyrogens develop they can be removed by filtration through a St. No. 3 pad, the pad in this case acting as an adsorbing agent. It has been shown that continued filtration of a pyrogenic solution through the pad will result in pyrogens reappearing in the solution. Two liters of a pyrogenic solution could be filtered, and rendered lower in pyrogen by passage through filter pads approximately 22 cm. in diameter. Table I shows the results obtained in such an experiment when a standard pyrogen test was carried out. Ten cc. of isinglass solution per kilogram were administered to each of five rabbits.

TABLE I

REMOVAL OF PYROGENS BY FILTRATION

	Temperature rise, ° F.	
	Average	Highest
Original solution	1.6	2.5
1st liter through pad	0.3	0.4
6th liter through pad	1.6	1.9
17th liter through pad	1.6	2.4

This process has been repeated on several occasions and whenever pyrogens have been encountered they have been successfully removed by such treatment.

Isinglass solutions have been stored for months without change in pyrogenicity. Two solutions prepared in March and retested in October gave the results shown in Table II.

TABLE II

THE EFFECT OF SEVERAL MONTHS' STORAGE AT 45° F. ON THE PYROGENICITY OF ISINGLASS

		Temperature rise, ° F.	
		Average	Highest
Solution 1			
March	Original solution	0.2	0.3
October	Retest	0.5	1.0
Solution 2			
March	Original solution	0.2	0.7
October	Retest	0.2	0.8

In all, about one hundred pyrogen tests were carried out before the process described was considered suitable for the preparation of isinglass solutions. No disturbances in the rabbits were noticed excepting the hyperthermia in cases where pyrogens were involved.

Discussion

The procuring of fresh frozen sounds was found to be essential if pyrogens were to be kept at a minimum in the preparation of isinglass. The dried sounds previously used for the preparation of isinglass could not be used when the purification by acetone precipitation was omitted. No trouble was found using tap water for the calcium hydroxide solution and the first two hydrochloric acid treatments, but in subsequent parts of the process the use of pyrogen-free water was found essential. Water was freshly distilled into 20-liter bottles, autoclaved at 120° C. for two hours and refrigerated. Water prepared in this manner could be stored until it was required for the washing of various pieces of equipment prior to sterilization by steaming. In the washing of the filter press approximately 40 liters of water were used. During the adjusting of acidity and determination of chlorides and solids content, the isinglass mixture was maintained at 70° C. as an effective means of maintaining sterility and avoiding the formation of pyrogens. In order to avoid changing the pads during the final filtration a press holding 30 pads was used. The filtrate was returned to a kettle in which it was kept at 70° C. and from this was filled, using sterile technique, into the final containers. The 400-cc. bottles were all carefully washed and rinsed with pyrogen-free water, the tops covered with paper, and then autoclaved. It was only by taking such precautions that we were able to repeatedly prepare a solution suitable for use.

Acknowledgments

The author wishes to express his thanks to Dr. N. B. Taylor for having discussed this problem with him personally when this work was undertaken; to Dr. D. M. Young and Mr. G. G. Waters under whose supervision pyrogen testing was carried out; and to Dr. G. A. McVicar for valuable suggestions during the course of this investigation.

Thanks are expressed to Mr. W. H. Brown for his technical assistance in planning and carrying out large scale operations.

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PROTECTION BY KILLED VOLE BACILLUS VACCINE AGAINST EXPERIMENTAL TUBERCULOSIS IN GUINEA PIGS[1]

By J. C. Paterson, D. W. Crombie, and J. C. Coles

Abstract

A vaccine made from vole bacilli killed by ultraviolet light has been shown to confer a significant degree of protection against experimental tuberculosis in guinea pigs. The degree of protection was comparable to that obtained from BCG. The results of this single experiment suggest that further work on this material should be carried out.

Although BCG possesses definite immunizing properties against tuberculosis in man and in experimental animals, it has not been universally accepted by the medical profession. There are those (5) who claim that our knowledge of tuberculosis immunity is not complete enough to justify artificial immunization with living organisms. These same critics suggest that, although inoculation with BCG is apparently not immediately dangerous, organisms may survive for years and may gradually regain virulence. Recently, the U.S. Public Health Service (4) recommended that further research on BCG be carried out before it is licensed commercially, and that attempts be continued to develop a material containing only dead organisms.

Among other materials under development is a vaccine made from the vole bacillus. Wells (7), who discovered this murine type of tubercle bacillus in 1937, found that inoculation with viable organisms conferred a significant amount of protection against experimental tuberculosis in guinea pigs. This finding has been confirmed by others (3, 6,* 2). It is to be noted, however, that living vole bacilli were used in all of these experiments. Preliminary investigations by Crombie and Hambleton, in this laboratory, indicated that *killed* vole bacilli might also confer some degree of protection against experimental tuberculosis in animals. These preliminary studies have been elaborated upon, and the results of a large scale experiment in which the merits of killed vole vaccine and BCG are compared are reported in the present paper.

Material and Method

The series consisted of 150 guinea pigs, weighing from 350 to 550 gm. each, and of approximately an equal sex distribution. The animals were weighed individually, tagged, and segregated. The series was divided into three lots, each lot having the same number of animals, and approximately the same average weight and sex distribution. The animals of Group 1 (50 guinea pigs) were inoculated with killed vole bacillus vaccine, Group 2 (50 guinea

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[1] *Manuscript received September 14, 1948.*

Contribution from the Queen Alexandra Sanatorium, Byron, Ont., and the Department of Medical Research, University of Western Ontario, London, Ont. Aided by a grant from the Mines Accident Prevention Association.

* *Quoted by Wells (7).*

pigs) with BCG, and Group 3 (50 guinea pigs) were put aside as controls. At an appropriate time, as described later, each animal in the three groups was infected with a large dose of a relatively avirulent strain of human tubercle bacilli. Following this challenge dose, the animals were observed daily, their survival times were noted, they were weighed at weekly intervals, and the sites of inoculation with vole vaccine and BCG were inspected for the presence of abscess formation. These measures were continued until all of the animals had died.

The vaccination with killed vole bacilli was carried out as follows: each of the guinea pigs in Group 1 was inoculated, by subcutaneous injection into the right groin, with two doses of vole bacilli killed by ultraviolet light. The doses were given at an interval of 28 days. The first dose consisted of 4 mgm. and the second of 1 mgm. moist weight of organisms. It was found by experiment that a one-minute exposure of a suspension (1 mgm. per cc.) of vole bacilli, 3 mm. deep in a porcelain plate, to a hot quartz ultraviolet light was sufficient to kill all of the organisms. However, for the actual vaccination, the exposure was increased to two minutes to ensure that all organisms were killed. Samples of the first vaccine were cultured at the time the material was injected, and these cultures were retained for four months when no growth was observed. The second vaccine, which was prepared in an identical fashion, was not cultured until 24 hr. after it was used. During this 24 hr. period the material stood at room temperature. No growth was observed from this second vaccine after four months.

The BCG used for the immunization of guinea pigs in Group 2 was obtained from the University of Montreal, and it was of a standard variety. Two doses of the material, each consisting of 10 mgm. moist weight of organisms, were injected subcutaneously, into the right groin of each animal. The doses were given at an interval of 28 days.

The challenge dose of tubercle bacilli was given by subcutaneous injection into the left groin, 28 days after the guinea pigs in Groups 1 and 2 had received their last dose of either killed vole vaccine or BCG. It consisted of 0.25 mgm. moist weight of a DT strain of human tubercle bacilli.* The actual suspension was prepared so that 1 cc. contained this weight of organisms. Suitable measures were taken to ensure that animals in the three groups received approximately the same amounts of infective material.

On the death of each animal an autopsy was carried out. Blocks of tissue were taken from the inguinal lymph nodes, from each lung at the level of the hilus, and from three different parts of the liver and spleen. The blocks were taken routinely from approximately the same location in each specimen, regardless of the site of the most striking gross lesions. The blocks of tissue were embedded in paraffin, and sections, cut at a thickness of 7μ, were stained with haematoxylin and eosin. The microscopic examination included an assessment of the number and size of individual tubercles in each organ as well as a description of the type of tuberculous process present.

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* *Although most workers have used very small doses (0.000001 mgm.) of a virulent strain of tubercle bacilli (1), we felt that by giving a larger dose of a less virulent strain greater accuracy in dosage could be obtained.*

Observations

A number of animals from each group died either before the challenge dose of tubercle bacilli was given or so soon afterward that the deaths could not be attributed, pathologically, to tuberculosis. These deaths from intercurrent infection have been eliminated from the series leaving the following number of animals in each group:

Group 1	(Killed vole vaccine)	44
Group 2	(BCG vaccine)	47
Group 3	(Controls)	45

All of the animals in each group ultimately died following infection with tubercle bacilli. The relative efficacy of the two types of vaccines was assessed by comparing the survival times, the loss in weight, and the extent of tuberculosis at autopsy, of animals in each group.

The average survival time for guinea pigs in the control group was 33 days; that for the killed vole vaccine group was 64 days; and that for the BCG group was 73 days. The average survival time in the two treated groups was, therefore, approximately twice that in the control group. The difference in survival times was particularly impressive at the 58th day of observation when all of the control animals had died while only 49% of the BCG group and 59% of the vole group had succumbed.

The individual survival times for the various animals are listed in Table I, and shown graphically in Fig. 1. They have been subjected to statistical analysis, using the standard error of difference to compare mean survival times, with the following results:

On comparing the control group versus the BCG group, the S.E. diff. = ±5.96. By calculating Fisher's t value the difference between these means is shown to be highly significant, P being less than 0.01.

Similarly, on comparing the control group versus the vole group, the S.E. diff. = ±4.17, and the difference is again shown to be highly significant, P being less than 0.01.

On the other hand, on comparing the vole group and the BCG group, the S.E. diff. = ±6.78, and the difference is not significant, P being between 0.2 and 0.1.

The weight loss, one month after infection with tubercle bacilli, was less marked in the treated animals than in the control animals. At this time, the average weight loss for the control animals was 10.9 gm., for the BCG animals it was 4.9 gm., and for the vole animals it was 3.7 gm.

TABLE I

INDIVIDUAL SURVIVAL TIMES OF GUINEA PIGS AFTER TUBERCULOUS INFECTION

Animal Number	Control group	BCG group	Vole group
1	14 Days	24 Days	30 Days
2	16 “	27 “	39 “
3	16 “	28 “	41 “
4	17 “	29 “	43 “
5	19 “	34 “	46 “
6	19 “	36 “	46 “
7	20 “	38 “	47 “
8	20 “	39 “	48 “
9	21 “	39 “	48 “
10	21 “	40 “	49 “
11	22 “	40 “	49 “
12	23 “	41 “	49 “
13	23 “	41 “	49 “
14	24 “	48 “	49 “
15	25 “	49 “	49 “
16	26 “	49 “	50 “
17	28 “	51 “	50 “
18	28 “	52 “	50 “
19	30 “	52 “	51 “
20	30 “	52 “	54 “
21	30 “	52 “	54 “
22	31 “	55 “	55 “
23	31 “	58 “	55 “
24	31 “	61 “	56 “
25	32 “	69 “	56 “
26	32 “	69 “	58 “
27	33 “	71 “	59 “
28	37 “	71 “	60 “
29	39 “	74 “	61 “
30	39 “	76 “	62 “
31	39 “	76 “	65 “
32	39 “	80 “	66 “
33	40 “	81 “	69 “
34	44 “	82 “	70 “
35	45 “	87 “	85 “
36	46 “	96 “	89 “
37	49 “	98 “	89 “
38	50 “	115 “	89 “
39	50 “	118 “	93 “
40	50 “	122 “	107 “
41	52 “	123 “	110 “
42	52 “	129 “	112 “
43	53 “	130 “	113 “
44	57 “	148 “	151 “
45	58 “	150 “	
46		151 “	
47		178 “	

Inflammatory reaction at the site of vaccination was less pronounced in the vole group than in the BCG group. More than half of the BCG injected animals showed abscesses of the groin, one month after vaccination, and many of these abscesses were large and were complicated by sinus formation. Only 4% of the vole injected animals had local reactions at this time, and in these the lesions were quite trivial.

Pathologically, widespread tuberculous lesions were found in the regional lymph nodes, liver, spleen, and lungs of almost every animal in the series. No significant difference in the number or severity of the lesions in members of the three groups could be detected; but in this regard it is to be noted that the treated animals lived twice as long as the controls, and the pathological findings are not, therefore, strictly comparable. Caseation of tubercles was more striking in the treated than in the control animals.

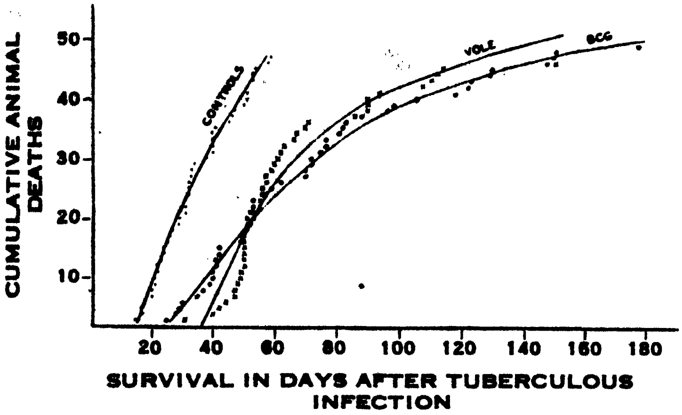

FIG. 1. *The cumulative animal deaths for the control group, the BCG injected group, and the killed vole vaccine group are plotted against the survival time in days.* • = *Controls.* ○ = *BCG.* × = *Vole vaccine.*

Discussion

The results of this large-scale experiment suggest that the vole bacillus, when killed by ultraviolet light and injected into guinea pigs, possesses definite immunizing properties against experimental tuberculosis in this species. The degree of protection afforded was comparable to that obtained from BCG. The efficacy of the killed material was as great as that of vaccines of living vole bacilli, as reported by others (3, 6*, 2).

Although the vole bacillus is said to be nonpathogenic to man (7), the advantages of a vaccine made from dead organisms only are obvious. It is recommended, therefore, that further experimental work be carried out on killed vole bacillus vaccine as an alternative to BCG, and to living vole vaccine, for human immunization. One refinement on our procedure would be to use either a smaller dose of infecting tubercle bacilli or a less virulent strain of organisms, and thus avoid the overwhelming infections that we achieved.

Acknowledgments

We are indebted to Miss Jean Millar for the statistical analyses reported in this paper.

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* *Quoted by Wells (7).*

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THE MUCOPOLYSACCHARIDES OF HUMAN SKIN[1]

By R. H. Pearce and E. M. Watson

Abstract

The mean values and standard deviations for the concentrations of the hyaluronic acid and chondroitin sulphuric acid fractions isolated from 11 specimens of human skin were 24.5 ± 5.7 and 26.2 ± 4.7 mgm. per 100 gm. of fresh tissue, respectively.

The individual differences in the concentrations of these substances were masked by the technical errors. The small variations in moisture and ash content of the skin did not contribute appreciably to the uncertainty of the concentration values. The purity of the products, determined by their amino sugar content, approximated 60%, which value compared favorably with those obtained by other workers.

The content of hexosamine and uronic acid identified both fractions as acid mucopolysaccharides. The release of reducing substances, including N-acetyl hexosamine, from the hyaluronic acid fraction by hyaluronidase paralleled the action of the enzyme upon hyaluronate from umbilical cord. The chondroitin sulphuric acid fraction, which was relatively resistant to the action of hyaluronidase, was assumed to be identical with the chondroitin sulphatelike substance that Meyer and Chaffee isolated from pig skin.

Introduction

The connective tissue, including the so-called ground substance, evidently participates in certain vital processes, in addition to exercising its wholly physical role in the provision of cohesion, support, and protection for the various cellular structures of the organism. Consequently, an active interest in the physiological and pathological significance of this tissue is developing (10). The present investigation is concerned with the mucopolysaccharides that are characteristic constituents of the connective tissue. The methods of analysis relating to these substances that have been evolved may facilitate investigations of their metabolism.

The mucoproteins, which are commonly referred to as mucins or mucoids, consist of saltlike combinations of a protein cation with a carbohydrate anion, the degree of their association being dependent upon the pH of the dispersing medium. The characteristic hydrophilic properties of the solutions from which such materials are separable appear to be related largely to the nature of the carbohydrate moiety. Therefore, the concentration of the so-called mucopolysaccharides in a tissue provides the best index of its mucinous nature.

As early as 1860, Rollett (18) reported that a constituent of a limewater extract of skin was precipitated with acetic acid and, because of the loss of tensile strength accompanying the extraction process, concluded that this

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[1] *Manuscript received September 15, 1948.*

Contribution from the Department of Pathological Chemistry, Faculty of Medicine, University of Western Ontario, London, Ont., with financial assistance from the National Research Council of Canada.

mucoprotein served as a cement substance. Van Lier (19) isolated a protein-free polysaccharide that contained sulphur, nitrogen, and either a pentose or a uronic acid from the skins of cattle, horses, dogs, and human beings. The presence of amino sugar in the mucoprotein derived from cow hide was demonstrated by Grassman *et al.* (5) and their data, as interpreted by Meyer and Chaffee (11), indicated also the presence of uronic acid.

Both Claude (3) and Chain and Duthie (2) isolated from rabbit skin a substance, the viscosity of which was reduced by the action of hyaluronidase. The latter workers noted also the release of N-acetyl hexosamine during the enzymatic hydrolysis. Meyer and Chaffee (11) isolated from pig skin two mucopolysaccharide fractions, one of which was identified as hyaluronic acid by the molecular ratio of nitrogen, glucosamine, glucuronic acid, and acetyl to titrable acid groups; by the optical rotation; and by the kinetics of its enzymatic hydrolysis. The other fraction contained nitrogen, galactosamine, glucuronic acid, and acetyl in the proportions found in chondroitin sulphuric acid but the specific rotation did not correspond with that of the latter compound. Bensley (1) showed by histological means the presence of a viscous mucinlike substance between the fibers of the corium of the guinea pig and Reuter (17) observed substances exhibiting the staining properties of the mucins in human skin.

Methods

The Estimation of the Cutaneous Mucopolysaccharides

Since no record of a critical investigation of the methods for the isolation of the mucopolysaccharides could be found, adequate criteria were not available to serve as a guide in the choice of a technique for the separation of these materials. Therefore, after preliminary trials of several procedures described by various writers, the method devised by Meyer and Chaffee (11) for the isolation of the mucopolysaccharides from pig skin was chosen as a basis for our work because of the high yield and excellent appearance of the products. This method was modified to provide a measure of the concentration of the mucopolysaccharides in human cutaneous tissue. A flow sheet illustrating the process is shown in Fig. 1.

The specimens of skin were obtained from legs that had been amputated surgically. The skin was carefully dissected from the limb as soon as possible following the operation with the inclusion of a minimum of subcutaneous fat. Areas exhibiting any gross pathological lesions were excluded from the sample. If the analysis could not be performed immediately, the tissue was stored in acetone at 4° C. Each specimen was ground in a Latapie mill using a known and approximately equal volume of distilled water as a lubricant.

An equal portion of *N* sodium hydroxide was added to the homogeneous paste. The resulting mixture was shaken three or four times daily during its incubation at 37.5° C. At the termination of the 48 hr. extraction period, the tissue had disintegrated almost completely yielding a slightly opalescent reddish-brown solution. Glacial acetic acid was added dropwise, with stirring, until the pH reached 7.0. The mixture was then clarified with Hyflo Supercel and vacuum filtration.

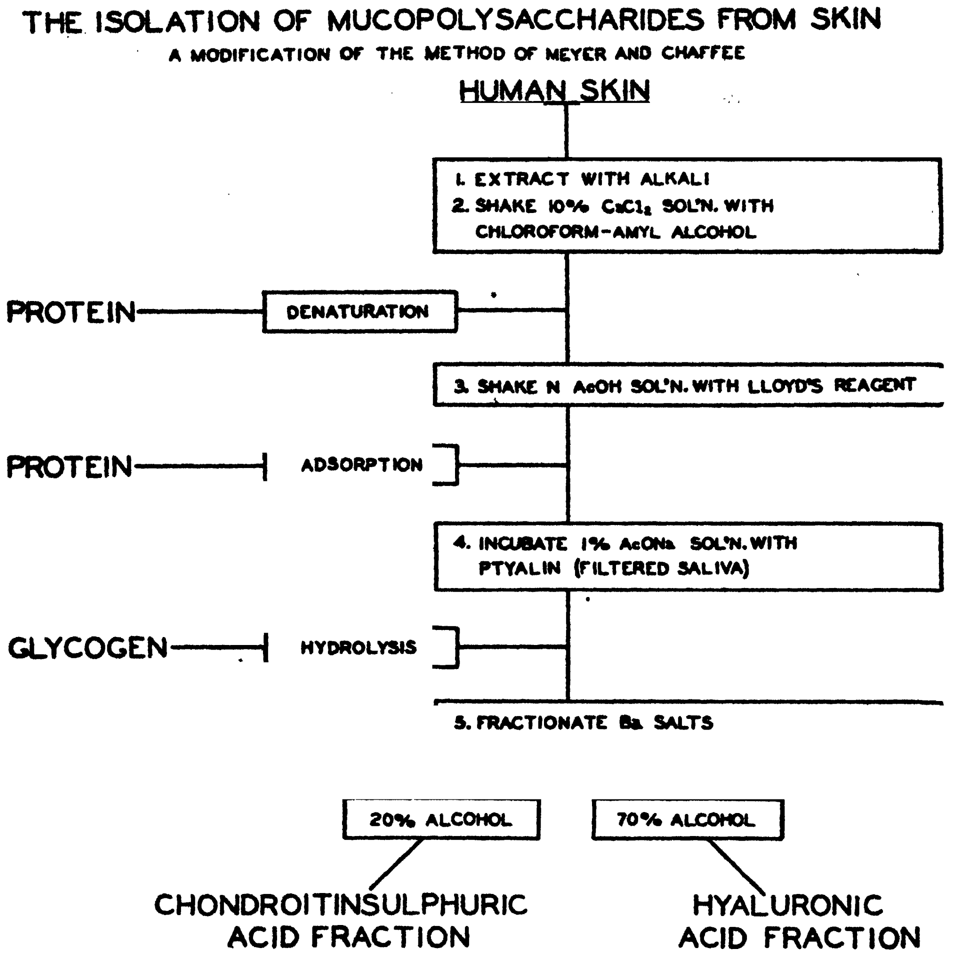
THE ISOLATION OF MUCOPOLYSACCHARIDES FROM SKIN
A MODIFICATION OF THE METHOD OF MEYER AND CHAFFEE
HUMAN SKIN
1. EXTRACT WITH ALKALI
2. SHAKE 10% CaCl2 SOL'N. WITH CHLOROFORM-AMYL ALCOHOL
PROTEIN
DENATURATION
3. SHAKE N AcOH SOL'N. WITH LLOYD'S REAGENT
PROTEIN
ADSORPTION
4. INCUBATE 1% AcONa SOL'N. WITH PTYALIN (FILTERED SALIVA)
GLYCOGEN
HYDROLYSIS
5. FRACTIONATE Ba SALTS
20% ALCOHOL
70% ALCOHOL
CHONDROITINSULPHURIC ACID FRACTION
HYALURONIC ACID FRACTION

FIG. 1. *A flow diagram for the isolation of the mucopolysaccharides from the skin.*

A boiling water bath was used to concentrate the extract until it became quite viscid; then the mucoprotein was precipitated by transferring the fluid with a minimal amount of wash water to two volumes of a mixture of 95% ethanol containing 1% (w/v) of potassium acetate and 1% (v/v) of glacial acetic acid. This precipitation mixture was used in all the separations described below.

The acid minimized the dissociation of the mucopolysaccharides, while the acetate probably neutralized the micellar charge. These actions, together with the dehydrating effect of the alcohol, produced a dense, easily separable product. Although the flocculation was apparently complete after the mixture had stood for two to three hours at 4° C., the precipitate could be obtained more readily when it had been left in the refrigerator over night.

The pale brown, gelatinous residue that separated upon centrifugation was dispersed in 50 ml. of 10% (w/v) calcium chloride solution. Following the addition of 10 ml. of a 4 : 1 mixture of chloroform and amyl alcohol and shaking for 48 hr., the denatured protein gel was centrifuged down and the slightly turbid supernatant was poured into 100 ml. of the precipitation mixture. In order to remove the last traces of protein, the white fibrinlike precipitate that formed was centrifuged off and dispersed in 20 ml. of distilled water. The latter was made one normal with respect to acetic acid by the addition of 1.2 ml. of the glacial acid. About 1 gm. of Lloyd's reagent was added and, after a thorough mixing, the suspension was centrifuged and the centrifugate was poured into 60 ml. of the precipitation mixture. The residue was washed with 10 ml. of *N* acetic acid and this centrifugate was also added to the precipitation mixture.

The white asbestoslike fibers of the separated mucopolysaccharides were dissolved in 10 ml. of a 1% (w/v) solution of sodium acetate to which 0.4 ml. of filtered saliva and five drops of toluene were added. The tightly corked tube was incubated subsequently for six or seven hours; then its contents were poured into 20 ml. of the precipitation mixture. This step eliminated any glycogen that may have been present.

After being dissolved in 10 ml. of 10% (w/v) barium acetate solution, the purified mucopolysaccharides were fractionated in the following manner. The barium acid chondroitin sulphate was precipitated by the addition of 2 ml. of 95% ethanol, which brought the alcohol concentration up to 20%, and the container was placed in a refrigerator over night. The barium hyaluronate was separated from the centrifugate by adding the latter to 32 ml. of the precipitation mixture.

The resultant barium salts of the mucopolysaccharides were separated by means of a centrifuge and dissolved in 10 ml. of distilled water. The chondroitin sulphate fraction usually gave a turbid solution, whereas the hyaluronate fraction was water-clear. A 1 or 2 ml. aliquot was removed with a pipette for subsequent analytical investigation and the remainder of the fluid was poured into 20 ml. of the precipitation mixture. The reprecipitated mucopolysaccharide was washed by centrifugation with 95% ethanol and transferred to a prepared filter paper that had been dried over night in an oven at 110° C. and kept in a desiccator over anhydrous calcium chloride prior to being weighed by the method of short swings. The residue on the paper was thoroughly washed three or four times with both ethanol and ether. After air-drying, the paper was placed *in vacuo* over anhydrous calcium chloride over night. The air was admitted to the desiccator through concentrated sulphuric acid and the paper containing the sample was weighed as before. The weighing was repeated at 24-hr. intervals until the readings were constant within 5%.

The weight of the mucopolysaccharide was corrected for the removal of the aliquot and for its theoretical barium content. The concentration of the acid mucopolysaccharide was expressed in relation to the fresh weight of the skin.

The Moisture and Ash Content of the Skin

Samples of the homogenized skin were dried *in vacuo* over anhydrous calcium chloride and phosphorus pentoxide until the weight remained constant within 0.3%. The dried tissue was charred carefully over a Bunsen burner and ignited at 550° C. in an electric oven for several hours until its loss in weight, after cooling in a desiccator, was less than 4%. The weight of the skin in the sample was calculable from the proportions of water and tissue used in the process of grinding.

The Determination of Hexosamine

First Procedure.—To a suitable aliquot of a solution containing the mucopolysaccharide, concentrated hydrochloric acid was added to bring its concentration to 4 *N*. The ampoule containing the mixture was evacuated and the polysaccharide was hydrolyzed by placing it in an oven at 110° C. for eight hours. The excess acid was 98 to 99% neutralized by slowly titrating the hydrolyzate with 7.5 *N* sodium hydroxide to a pH between 2.5 and 5.0, with constant stirring. The contents of the ampoule were then transferred to a 10 ml. volumetric flask, diluted to volume, and kept at 4° C. until ready for analysis. While this technique avoided the liberal dilution associated with the hydrolytic procedure employed by Palmer *et al.* (16), a considerable error was introduced as a result of the difficulties of the microtitration and the destruction of hexosamine by localized excesses of alkali.

The amino sugar content of the hydrolyzate was determined by the method of Palmer *et al.* (16), modified somewhat for use with the Cenco-Sheard-Sanford Photelometer and with the employment of an Evelyn 565 mμ light filter.

Second Procedure.—The method of Palmer *et al.* (16) was used for the hydrolysis and the color development but with the additional sensitivity provided by the Beckman spectrophotometer. The optical densities were read at 530 mμ.

The Determination of Acetyl Hexosamine

The method of Morgan and Elson (15) for the determination of acetamidohexoses was adapted for use with the Cenco-Sheard-Sanford Photelometer. An Evelyn 565 mμ light filter was employed.

The Determination of Uronic Acid

The technique of Maughan, Evelyn, and Browne (9) was modified so that the light transmission could be read with a Cenco-Sheard-Sanford Photelometer at 565 mμ.

The Determination of Reducing Substances

First Procedure.—The method of Folin and Malmros (4) was adapted for use with the Klett-Summerson photoelectric colorimeter. The transmission band of the 660 mμ light filter coincided more closely with the wave length of maximal adsorption and it was consequently more suitable for this analysis than the 540 mμ filter recommended by the manufacturer (7). The gum ghatti in the color reagent was unnecessary for stabilizing the ferric ferrocyanide suspension at the concentrations of glucose that were being measured. The light transmission reached a minimum 20 min. after the addition of the color reagent and was read at this time.

Second Procedure.—The sensitivity of the Folin-Malmros (4) method was increased sufficiently for use with the Cenco-Sheard-Sanford Photelometer by avoiding the dilution prior to the addition of the color reagent. A Klett 540 mμ filter was used.

The Colorimetric Technique

A standard solution of the substance being determined and a blank were run with each batch of test solutions so that a calibration curve could be constructed for each group of analyses. Every procedure was investigated carefully to determine the concentration range in which Beer's law applied, as well as the expected error.

TABLE I

THE CONCENTRATION OF THE MUCOPOLYSACCHARIDE FRACTIONS IN HUMAN SKIN

Specimen No.	Wt. of fresh skin, gm.	Sex	Age, yr.	Hyaluronic acid,[*] mgm./100 gm.	Chondroitin sulphuric acid,[*] mgm./100 gm.	H/C ratio
86	232	M	73	26.0	24.6	1.06
87	220	M	79	29.4	24.0	1.23
88	121	M	64	34.6	31.0	1.12
89	131	F	62	14.3	27.4	0.52
90	121	F	70	19.8	24.0	0.82
91	118	F	70	26.2	29.0	0.90
92	236	M	71	18.8	18.8	1.00
93[**]	255	—	—	21.6	31.8	0.68
212	124	F	77	23.0	29.2	0.79
226	184	M	69	27.7	18.2	1.52
231	143	M	86	27.9	30.5	0.92
Mean				24.5	26.2	0.96
Standard deviation				5.7	4.7	0.27
Standard error				1.7	1.4	0.08

[*] *The mucopolysaccharides were isolated and weighed as their barium salts. The yields were corrected for the removal of an aliquot for analysis and the concentrations were expressed as the amount of the free acid in the fresh skin.*

[**] *This specimen of skin was obtained from an arm.*

Results

The Concentration of the Cutaneous Mucopolysaccharides

Samples of skin obtained from 10 legs amputated above the knees because of gangrene of the feet were analyzed in the manner described above. A specimen secured from an arm was also included. The concentrations of the cutaneous mucopolysaccharides in these specimens are given in Table I. The relative frequency at which the various concentrations of each substance were observed has been demonstrated by grouping the data under two sets of class intervals in order to provide a better over-all picture of the distribution than that obtainable from a simple histogram. The results for the hyaluronic and chondroitin sulphuric acid fractions are presented graphically in Figs. 2 and 3, respectively.

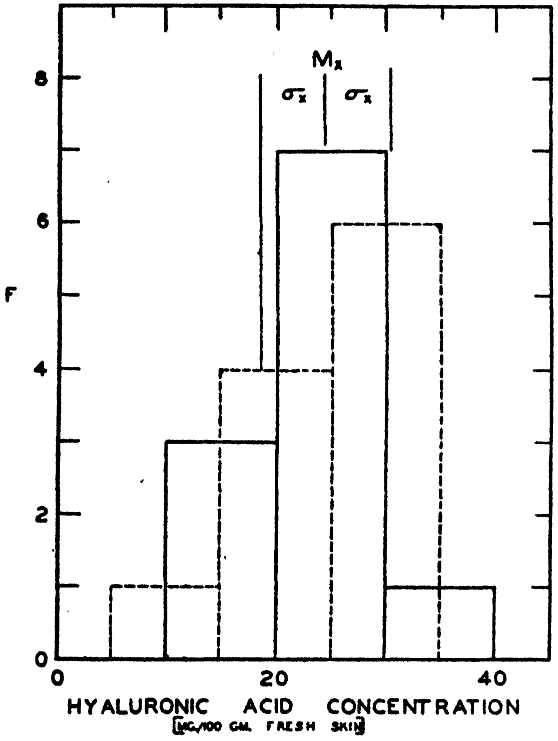

FIG. 2. *The frequency distribution of the hyaluronic acid concentrations in human skin. The data are grouped under two sets of class intervals.*

The Relationship of Age and Sex to the Cutaneous Mucopolysaccharides

The observed values were surveyed with a view to deciding whether or not the sex or the age of the individuals bore any relationship to the concentration of the cutaneous mucopolysaccharides. The results of the analyses of the skin from the legs of six men and four women were considered. The mean concentrations for each of these groups of individuals, with the associated standard errors, may be seen in Table II.

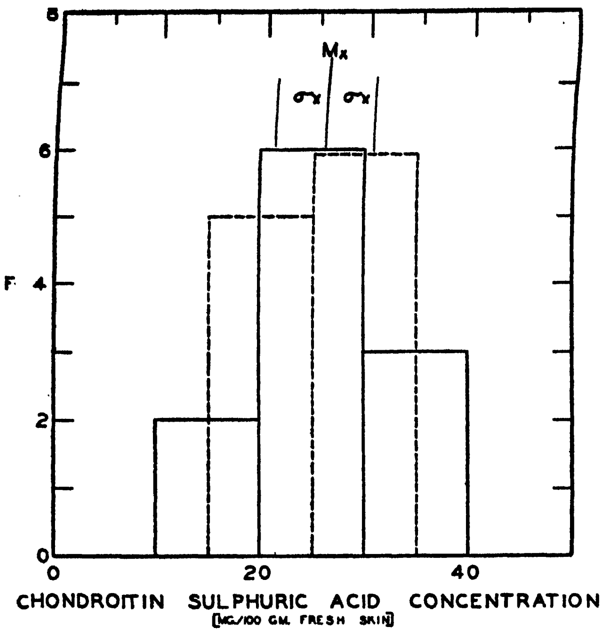

FIG. 3. *The frequency distribution of the chondroitin sulphuric acid concentrations in human skin. The data are grouped under two sets of class intervals.*

TABLE II

THE RELATIONSHIP OF THE SEX OF THE INDIVIDUAL TO THE CUTANEOUS MUCOPOLYSACCHARIDE CONCENTRATIONS*

Sex	No. of samples	Hyaluronic acid, mgm./100 gm.	Chondroitin sulphuric acid, mgm./100 gm.	H/C ratio
Male	6	27.4 ± 2.1	24.5 ± 2.2	1.14 ± 0.09
Female	4	20.8 ± 2.5	27.4 ± 1.2	0.76 ± 0.08
	P	0.07	0.36	0.019

* *Calculated from the data presented in Table I. The means are given with their standard errors.*

The Reproducibility of the Method

The limited amount of normal skin that was obtainable from any single specimen did not permit multiple analyses with the provision of yields sufficient for accurate weighing. Consequently, the error inherent in the method was assessed by analyzing the material derived from the grossly thickened skin from an amputated myxoedematous leg. This skin was treated in the same manner as the control specimens of skin referred to above except that, after the tissue was ground, it was further diluted with an equal volume of water so that the mucopolysaccharide concentration would be of approximately the same order as that occurring in the control specimens. Three samples of the same specimen were carried through the isolation processes individually. The data shown in Table III were obtained.

TABLE III

THE REPRODUCIBILITY OF THE YIELDS OF MUCOPOLYSACCHARIDES

Specimen No.*	Hyaluronic acid**, mgm./100 gm.	Chondroitin sulphuric acid**, mgm./100 gm.
229-2	. 57.1	47.2
229-1	60.9	48.4
229-3	72.9	50.6
Mean	63.6	48.7
S.d.	8.2	1.7

* *The skin from a patient with pretibial myxoedema was analyzed.*
** *The data were calculated in the same manner as those in Table I.*

Furthermore, two groups of control specimens that were analyzed 17 months apart yielded almost identical values, thus providing an additional indication of the reproducibility of the analytical technique.

The Moisture and Ash Content of the Skin

Since the concentration of the mucopolysaccharides in the skin has been expressed in terms of the fresh weight of the tissue, one might suspect that a large proportion of the variability of the results are explainable by differences in the amounts of water and inorganic materials in the skin of different individuals. The extent to which such factors influenced the results was judged by determining the moisture and ash content of four specimens of skin that were undergoing analysis. The observed values are given in Table IV.

TABLE IV

THE MOISTURE AND ASH CONTENT OF HUMAN SKIN

Specimen No.	Moisture, %	Ash, %
230	61.8	1.08
225	60.8	0.72
231	61.4	0.36
226	60.2	0.78
Mean	61.0	0.74
S.e.	0.4	0.15

The Purity of the Products

The determination of the amino sugar content of the mucopolysaccharides provided a simple measure of the purity of the isolated fractions, since hexosamine is a characteristic component of the molecule and its theoretical concentration can be calculated. The results of such a determination are recorded in Table V. The low values that were obtained were attributable in part at least to the destruction of the hexosamine during the neutralization of the hydrolyzate. A proportion of the error in the estimate of purity doubtless could be ascribed to the uncertainties in the values for both the concentration of hexosamine and the weight of the sample.

TABLE V

THE PROPERTIES OF THE MUCOPOLYSACCHARIDE PREPARATIONS

Property studied	Hyaluronate		No. of samples	Chondroitin sulphate		No. of samples	*P*
	Mean	S.d.		Mean	S.d.		
Hexosamine content as % of theory*							
1st series	60	16	11	60	13	10	—
2nd series	79	10	4	54	5	4	—
Enzymatic hydrolysis as % of theory							
1st series** (*a*)	69	17	11	−1	3	11	<0.001
(*b*)	145	50	11	−2	6	10	<0.001
2nd series*** (*a*)	11.5	1.8	3	3.7	1.1	3	<0.001
(*b*)	15.2	3.6	3	7.4	1.9	3	0.011

* *Determined by comparing the estimated hexosamine content with the theoretical value.*

** *Estimated by the release of N-acetyl hexosamine by hyaluronidase at pH 6.0 and calculated (a) from the weight and (b) from the observed hexosamine content of the preparations.*

*** *As indicated by the release of reducing substances through hyaluronidase action at pH 6.0 in 0.3 M sodium chloride and expressed as for the first series.*

Another series of preparations was analyzed by the more accurate "second procedure" described above. The values that were obtained (see Table V) indicated that the products contained some contaminating material.

As a further check of the general efficacy of the isolation technique, a specimen of hyaluronic acid and of calcium acid chondroitin sulphate, each prepared in this laboratory, were subjected to analysis and the results compared with those that Meyer (10, 12, 13) had obtained under similar conditions.

The Response of the Mucopolysaccharide Preparations to Hyaluronidase

A diluted portion of the aliquot retained for analysis was added to an equal volume of 0.1 *M* acetate buffer, pH 6.0, containing 1 mgm. of hyaluronidase, i.e. approximately seven turbidity reducing units, per ml. The mixture was divided into two parts, in one of which the enzyme was inactivated by raising the temperature to 100° C. for 30 min. The other was incubated for 24 hr. at 37.5° C. in the presence of toluene and the enzyme was destroyed subsequently by heating. Aliquots were removed from each of these mixtures and their content of free N-acetyl hexosamine and uronic acid was estimated.

The theoretical amount of N-acetyl hexosamine present was calculated on the basis of the yield of the mucopolysaccharide that had been isolated, using the molecular weights of the moieties deduced to be present in the molecule. By comparing this theoretical amount of acetyl hexosamine with the amount released by the enzyme, an estimate of the degree of hydrolysis was obtained (see Table V). The errors both in the estimated concentration of acetyl hexosamine and in the weight of the sample accounted for a large part of the error in the observed values. The highly significant difference in the responses of the hyaluronate and chondroitin sulphate fractions is indicated by a '*t*' value of 12.7.

Since these mucopolysaccharides had been found to be quite impure, a better estimate of the degree of enzymatic hydrolysis was sought by correcting the value based upon the weight of the sample for the hexosamine content of the mucopolysaccharide preparations. The results of these computations are given in Table V. The considerable error in this corrected degree of hydrolysis originated from the errors involved in the estimates of both the purity and the degree of hydrolysis.

The results of the analyses for uronic acid in the same solutions could not be interpreted on a quantitative basis. However, the color that was produced in each test provided qualitative evidence of the presence of uronic acid in the molecule.

In view of the anomalous results that were obtained with the above technique, the extent of enzymatic hydrolysis of a second series of preparations was estimated by a slightly different procedure. The strength of the acetate buffer was doubled and sodium chloride was added to a concentration of 0.6 *M* in order to eliminate the inhibition of the enzyme, which is known to occur in the absence of these ions. The quantity of glucose equivalent to the reducing substances that were released by the enzyme was determined by the "first procedure." The amount equivalent to a complete hydrolysis was calculated from the data of Meyer, Smyth, and Dawson (14) and the degree of hydrolysis computed therefrom. This value was corrected further for the impurities present as determined by analyses for hexosamine. These data may be found in Table V.

The Kinetics of the Enzymatic Hydrolysis

A specimen of the cutaneous hyaluronate fraction and a specimen of hyaluronic acid prepared by the same procedure from umbilical cord were dissolved in McIlvane's buffer, pH 6.0, at a concentration that approximated 0.4 mgm. per ml. The quantity of mucopolysaccharide that was present in each solution was estimated by its content of hexosamine using the "first procedure." Samples from each of these solutions were added to equal portions of a series of diluted solutions of hyaluronidase in 0.1 *M* acetate buffer, pH 6.0. After incubation for 2 and 20 hr. at 37.5° C., aliquots were removed from the mixture for the estimation of reducing substances by the "second procedure." The degree of hydrolysis was calculated from the proportion of the total theoretical amount of reducing substances that had been released. The results of these calculations are presented in Fig. 4.

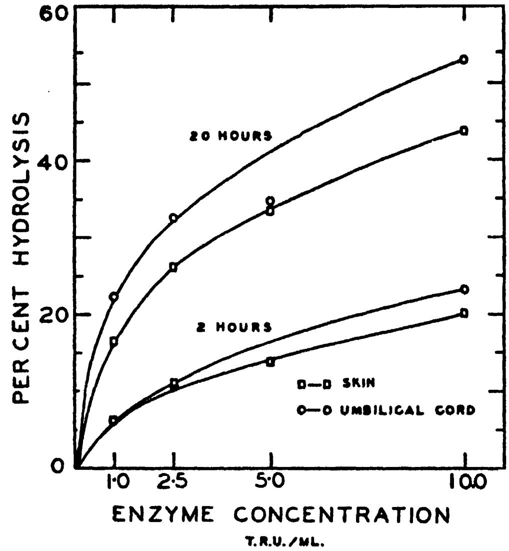

FIG. 4. *The kinetics of the hydrolysis of the cutaneous mucopolysaccharide and of the hyaluronate from umbilical cord at pH 6.0 and 37.5° C.*

Discussion

Biochemical studies of the mucopolysaccharides have been hampered by a lack of appropriate analytical methods. Therefore, the results of an investigation of this kind can be interpreted only in the light of the limitations thus imposed.

In so far as may be judged from the data presented, the proposed modification of the method of Meyer and Chaffee (11) for the isolation of the mucopolysaccharides from pig skin provides an index of the concentration of these components in human skin.

While the products obtained by this procedure were not pure, the degree of their contamination was uniform and therefore did not contribute an appreciable error to the results. The amount of impurities detected was not extraordinary considering the known colloidal properties of the mucopolysaccharides and the complex character of the original material. Indeed, the increase in the concentration of the substances sought from 0.03% in the fresh skin to 60% in the isolated product provided a favorable indication of the efficacy of the isolation process. The degrees of purity of the mucopolysaccharides prepared in this laboratory were almost identical with those of Meyer's preparations (10, 12, 13).

The standard deviation observed in triple analyses of a single specimen of skin was greater than that found in a series of analyses. Consequently, the error connected with the analytical technique *per se* must have masked any individual differences in concentration. Since the ratio of the yields of the two fractions was more constant than the yields themselves, this error must have originated principally in the separation processes rather than in the fractionation procedure. The greater consistency of the yields of the chondroitin sulphate fraction, as compared with those of the hyaluronate fraction, probably resulted from the more specific means for its separation.

The remarkably constant moisture and ash content of the skin clearly indicated that the expression of the results in terms of the fresh weight of the skin did not diminish appreciably their reproducibility.

While the exact nature of the products could not be established definitely, the presence of N-acetyl hexosamine and uronic acid pointed to these substances being acid mucopolysaccharides. The concentration of hexosamine was sufficiently close to theory to support this suggestion.

The one fraction closely resembled hyaluronic acid as regards the extensive release, by the action of hyaluronidase, of reducing substances, including a product that gave the color reaction of N-acetyl hexosamine. The kinetics of this hydrolysis were paralleled by those of an hyaluronate preparation from umbilical cord. The high value for the extent of the apparent hydrolysis as determined by the release of N-acetyl hexosamine was, at first, extremely puzzling. However, since the completion of these analyses, Humphrey (6) has shown that an intermediate breakdown product of hyaluronic acid gives an intense color identical with that produced by N-acetyl hexosamine, so that an apparent N-acetyl hexosamine content approximately double the expected value is possible. The most likely explanation for this phenomenon is that the N-acetyl hexosamine is transformed by the analytical procedure only partially into the supposed cyclic condensation product responsible for the color development, whereas this condensation product is released as such from the hyaluronic acid upon enzymatic hydrolysis. No substance other than hyaluronic acid is known to possess this property.

The other fraction could not be identified as accurately as that referred to above. However, the observed data coupled with the knowledge that Meyer and Chaffee (11) isolated hyaluronic acid and a chondroitin sulphuric acid-like substance from pig skin have led to the tentative opinion that the two fractions in question were identical with those obtained by these investigators.

The concentrations of the mucopolysaccharides in the skin from human legs, as determined by this technique, may, of course, only approximate the true values since there is no proof that their separation was complete.

The distribution of the observed values was accounted for entirely by the deficiencies of the analytical procedures, leading to the conclusion that the individual differences in the concentrations must have been relatively small. The frequency distribution of the observed concentrations was sufficiently close to normal to warrant statistical comparisons based upon the Gaussian curve of error.

While the age of the individual bore no apparent relationship to the cutaneous mucopolysaccharide concentrations, there seemed to be less hyaluronic acid in the skin of the females that were examined than in that of the males. This difference was masked by the variability of the observed concentrations but became apparent when the ratio of the two components was considered. This observation is in accord with the lesser intracutaneous resistance of female rabbits as compared with males (8).

Summary

1. An adaptation of a method described by Meyer and Chaffee (11) for the isolation of the mucopolysaccharides from pig skin was utilized for the quantitative estimation of the mucopolysaccharides in human skin.

2. By this means, the concentration of hyaluronic acid and of another acid mucopolysaccharide, probably chondroitin sulphuric acid, in 11 samples of normal skin was found to be 24.5 ± 5.7 and 26.2 ± 4.7 mgm. per 100 gm. of fresh skin, respectively. (These are standard deviations.)

3. Less hyaluronic acid was observed in the skin of women than in that of men.

Acknowledgments

The work reported herein was presented in part before the Division of Biological Chemistry at the 111th National Meeting of the American Chemical Society, in Atlantic City, N.J., Apr. 14, 1947. Permission for the publication of the data has been granted.

The hyaluronidase employed in this investigation was supplied through the kindness of Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N.J., U.S.A.

Mr. J. Charles Honey of the Canadian Johns-Manville Co., Ltd., Toronto, provided a generous supply of Hyflo Supercel.

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 27, SEC. E. APRIL, 1949 NUMBER 2

HÉPATECTOMIE PARTIELLE ET RÉSISTANCE AUX BRÛLURES

V. VARIATIONS DE L'ACIDE ASCORBIQUE ET DU POIDS DES SURRÉNALES[1]

PAR L. P. DUGAL ET A. DESMARAIS

Abstract

The changes in adrenal ascorbic acid and weight have been studied in four groups of animals: (1) partially (65%) hepatectomized rats, (2) partially hepatectomized ones, burned six hours after operation, (3) sham operated controls, and (4) sham operated controls burned six hours after operation. Results show slight but significant differences in ascorbic acid concentration and adrenal weight between Group 2, in which the mortality is higher, and the other groups: ascorbic acid concentration is a little lower in the first 24 hr. after burns, and adrenal weight becomes higher only 48 hr. after burning.

Dans des travaux précédents, nous avons montré que l'hépatectomie partielle diminue la résistance du rat blanc aux brûlures (4). Afin de trouver une explication à ce phénomène, nous avons entrepris d'étudier la régénération du foie après les brûlures (5), l'influence du jeûne sur la mortalité des animaux partiellement hépatectomisés et brûlés (article sous presse), les variations de la glycémie (2 et article sous presse) et de l'azote-α-aminé du sang total (3).

Le présent travail est consacré à l'étude des variations de l'acide ascorbique et du poids des surrénales chez des rats blancs partiellement hépatectomisés et leurs témoins opérés à blanc, brûlés et non brûlés.

Protocole expérimental

Des lots de rats albinos, tous mâles et de poids équivalents (125 à 145 grammes) ont été divisés en quatre groupes: (1) témoins opérés à blanc, non brûlés; (2) témoins opérés à blanc et brûlés; (3) hépatectomisés (65%), non brûlés; (4) hépatectomisés (65%) et brûlés. Six heures après les opérations, les animaux des groupes 2 et 4 furent brûlés suivant une méthode standard que nous avons déjà décrite (4).

L'extraction et les dosages de l'acide ascorbique ont été effectués par la méthode de Bessey et King (1), à des intervalles de 0, 6, 12, 24, 48, 72, 96 et 120 heures après les brûlures, et à des périodes correspondantes pour les non

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[1] *Manuscrit reçu le 30 novembre 1948.*
Contribution du Département de Recherches en Acclimatation, Institut d'Hygiène et de Biologie Humaine, Faculté de Médecine, Université Laval.

brûlés. Les surrénales, prélevées sous anesthésie à l'éther, étaient d'abord déposées sur un papier filtre, soigneusement débarrassées du tissu adipeux adhérent, pesées, puis déposées dans le mélange d'extraction. L'ensemble de ces opérations ne dure pas plus que quatre minutes.

Les résultats que nous allons exposer sont basés sur des moyennes obtenues des dosages et pesées effectués sur les surrénales de 10 animaux au moins, soit, pour toute l'expérience, près de 400 animaux.

Résultats

(1) *Acide ascorbique* (mg. par gr. de tissu frais)

La concentration normale de l'acide ascorbique dans les surrénales, chez les animaux de 135 à 145 grammes, est de 4.60 ± 0.34 mg.

Les résultats obtenus apparaissent dans le tableau ci-joint. Nous voyons que la concentration diminue au cours des six heures qui suivent l'opération: l'abaissement est de 35.8% de la valeur normale chez les témoins et de 33.4% chez les hépatectomisés. Au moment des brûlures (point 0), hépatectomisés et témoins ont donc des concentrations d'acide ascorbique sensiblement identiques.

TABLEAU I

I. Acide ascorbique (mg. par gr. de tissu frais). Normale: 4.60 ± 0.34*
II. Poids des surrénales (en mg.). Normale: 14.6 ± 1.5

Heures après les brûlures	Témoins non brûlés		Témoins brûlés	
	I	II	I	II
0	2.95** ± 0.19	24.9 ± 1.7	2.95** ± 0.19	24.9 ± 1.7
6	3.85 ± 0.28	25.5 ± 3.0	3.31 ± 0.30	29.3 ± 2.3
12	3.80 ± 0.30	29.6 ± 2.4	3.45 ± 0.29	31.6 ± 2.4
24	3.67 ± 0.26	27.5 ± 2.3	2.86** ± 0.26	29.0 ± 2.4
48	4.03 ± 0.34	28.1 ± 2.1	4.78 ± 0.41	32.1 ± 2.4
72	4.12 ± 0.36	29.4 ± 2.2	3.54 ± 0.30	33.1 ± 2.6
96	3.88 ± 0.32	28.3 ± 3.1	4.04 ± 0.32	32.6 ± 2.6
120	4.04 ± 0.33	30.1 ± 2.2	4.14 ± 0.34	34.3 ± 2.7
	Hépatectomisés non brûlés		Hépatectomisés brûlés	
	I	II	I	II
0	3.06** ± 0.21	23.9 ± 1.7	3.06** ± 0.21	23.9 ± 1.7
6	3.69 ± 0.38	30.3 ± 2.4	2.93** ± 0.28	29.8 ± 2.3
12	3.58 ± 0.29	29.8 ± 2.4	2.77** ± 0.26	32.2 ± 2.6
24	3.33 ± 0.30	32.3 ± 2.4	3.11** ± 0.27	28.7 ± 2.2
48	4.48 ± 0.36	29.7 ± 2.2	4.25 ± 0.34	29.6 ± 2.3
72	4.25 ± 0.33	30.7 ± 2.2	3.85 ± 0.31	37.3 ± 3.2
96	4.27 ± 0.31	31.7 ± 2.3	3.84 ± 0.29	35.4 ± 2.9
120	3.89 ± 0.29	32.8 ± 2.4	3.72 ± 0.25	41.5 ± 3.0

* *Erreur probable.*

** *Ecarts de la normale statistiquement significatifs.*

A partir du point 0, la concentration augmente chez tous les groupes, sauf les hépatectomisés brûlés où l'abaissement se continue jusqu'à 12 heures après les brûlures, pour atteindre une valeur minima de 39.7%. Après 120 heures (cinq jours), les valeurs se sont progressivement rapprochées de la normale chez tous les groupes.

L'analyse statistique de ces résultats nous a montré que les moyennes obtenues sont très significatives; les valeurs de "*t*" sont toutes comprises entre 2.2 et 6.8, ce qui indique une probabilité de 95% et plus. Les différences entre les groupes, considérées à quelque moment que ce soit après les brûlures, sont peu considérables et n'ont pas de valeur statistique.

Il est intéressant de noter que le groupe des hépatectomisés brûlés est le seul chez lequel toutes les valeurs de la concentration, au cours des 24 premières heures après les brûlures, ont un écart de la normale statistiquement significatif.

Nos résultats, chez les animaux brûlés, confirment ceux de Ludewig (8) et de Long (6, 7).

(2) *Poids des surrénales*

Le tableau ci-joint (Tableau I) donne aussi les variations du poids des surrénales, exprimées en mg., chez ces mêmes animaux. Le poids de ces organes chez les animaux normaux témoins est de 14.6 ± 1.5 mg.

Nous constatons, dans les six heures qui suivent les opérations, une forte augmentation du poids des surrénales, à peu près identique chez les témoins et chez les hépatectomisés, soit 63.7% pour ces derniers et 70.1% pour les premiers. Après les brûlures, l'augmentation est moins rapide; à la fin de la période expérimentale, nous avons les valeurs suivantes, exprimant le poids acquis depuis le moment des brûlures (point 0):

Témoins non brûlés	37.2%
Témoins brûlés	37.7%
Hépatectomisés non brûlés	20.9%
Hépatectomisés brûlés	73.6%

Cette augmentation, qui dissocie des trois autres groupes celui des hépatectomisés brûlés, est acquise de 48 à 120 heures après les brûlures. Avant 48 heures, l'évolution est la même dans les quatre groupes, et c'est la période importante, puisque, nous l'avons déjà montré (4), c'est au cours des 48 premières heures après les brûlures que la mortalité est la plus forte chez les hépatectomisés brûlés.

Interprétation

Alors que nous nous attendions à des différences considérables dans le niveau de l'acide ascorbique et le poids des surrénales entre les hépatectomisés brûlés et les autres groupes, l'ensemble des résultats montre qu'il n'en est rien. Les seules différences observées entre les hépatectomisés brûlés et les trois autres groupes sont les suivantes: un abaissement un peu plus marqué et significatif de la concentration en acide ascorbique au cours des 24 premières heures après les brûlures; une plus grande augmentation du poids des surrénales, se produisant seulement 48 heures après les brûlures. Ces différences ne nous permettent pas d'apporter à notre problème une solution définitive.

L'ensemble de nos résultats soulève la question de la relation entre l'intensité du "stress" et celle de la réponse des surrénales. De plus, il est nécessaire de noter que, chez les hépatectomisés brûlés, la survie s'améliore à mesure que progresse la régénération hépatique. Si nous empruntons à nos travaux précédents les chiffres obtenus sur la survie des hépatectomisés (4) et la régénération du foie (5) après les brûlures, nous obtenons le tableau suivant, montrant que la mortalité régresse à mesure que le foie régénère.

Période, heures	Mortalité, %	Régénération, %
0 - 24	24.0	1.3
24 - 48	11.0	19.5
48 - 72	9.0	17.6
72 - 96	9.0	4.3
96 - 120	1.0	8.3

Tout se passe comme si l'intégrité de la fonction hépatique était nécessaire, soit pour assurer la capacité réactionnelle des tissus à la sécrétion corticale, soit pour activer les hormones corticales. Les récents travaux de Roberts et Szego (9) ont montré que le foie joue un rôle dans l'activation des oestrogènes, probablement en assurant la formation d'un composé soluble dans l'eau entre ces substances et une protéine d'origine hépatique. Le foie ne pourrait-il jouer un rôle semblable dans l'activation des stéroïdes corticaux? C'est l'hypothèse qui ressort de nos travaux sur l'hépatectomie partielle et la résistance aux brûlures. Les expériences en cours nous permettront de vérifier si une telle hypothèse est fondée, et peut-être d'éclairer d'un jour nouveau les relations entre la fonction hépatique et la réaction surrénalienne d'adaptation.

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LIPIDS OF THE NERVOUS SYSTEM DURING *IN VITRO* DEGENERATION[1]

BY A. C. JOHNSON, A. R. MCNABB, AND R. J. ROSSITER

Abstract

The changes in the concentration of lipids during the *in vitro* degeneration of cat brain slices and sciatic nerve have been determined after incubation in bicarbonate buffer for periods of time from 1 to 14 days. In brain slices incubated for 14 days there was a great decrease in the concentration of total phospholipid with no change in the concentration of cerebroside or total cholesterol. Of the individual phospholipids, there was a decrease in the concentration of sphingomyelin and cephalin and no significant change in the concentration of lecithin. In sciatic nerve incubated for eight days, there was a significant decrease in the concentration of cephalin and a decrease of borderline significance in the concentration of total phospholipid. There was no significant change in the concentration of cerebroside, free or total cholesterol, sphingomyelin, or lecithin. Thus in tissue of the nervous system, there appears to be a phospholipid-splitting mechanism, possibly enzymatic in nature.

Introduction

In previous reports on the changes in lipid concentration during the *in vitro* autolysis of tissue from the nervous system, brain only was used and only a limited number of the lipid components were estimated. In this investigation, the concentration of cerebroside, free and total cholesterol, total phospholipid, lecithin, sphingomyelin, and cephalin was determined during *in vitro* degeneration of both brain slices and peripheral nerve.

Changes in lipid content of nerves degenerating *in vivo* have been reported by Mott and Halliburton (16), May (14), Falk (4), Randall (19), and Johnson, McNabb, and Rossiter (8). Investigations on the lipids of cat sciatic nerve during Wallerian degeneration (Johnson *et al.* (8)) seemed incomplete without carrying out parallel *in vitro* studies. Monckeberg and Bethe (15), Nageotte (17), and Cajal (2) showed that the histological changes of nerves autolyzing *in vitro* were typical of those found in Wallerian degeneration.

As far as we know, there are no reports in the literature on the lipid content of autolyzed nerve. However, there are some data on the concentration of lipids of brain tissue during autolysis. Simon (21), Stamm (24), and Fries, Schachner, and Chaikoff (5) reported a hydrolysis of phospholipids during incubation. The most recent work is that of Sperry (22), who found a constant decrease in the concentration of total phospholipid in homogenates of rat brain. Jungmann and Kimmelstiel (10) reported that cerebroside decreased and inorganic phosphorus increased in autolyzing rabbit brain. None of the above workers estimated the individual phospholipids.

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[1] *Manuscript received January 7, 1949.*

Contribution from the Department of Biochemistry, University of Western Ontario, London, Ont. The research was supported by a grant from the National Research Council of Canada.

Methods

Autolysis of Brain

The brain of a cat was removed as soon as possible after death. With aseptic precautions brain slices were cut from each frontal lobe, one side providing the tissue for the control, the other side the sample for autolysis. The slices were transferred to two tared sterile Thunberg tubes, each containing 3 ml. of either Krebs bicarbonate buffer, pH 7.4, or bicarbonate buffer with isotonic saline. The Krebs bicarbonate buffer contained the cations, sodium, potassium, calcium, and magnesium, and the anions, chloride and phosphate, in approximately physiological proportions, while the only ions, other than bicarbonate, present in the bicarbonate buffer with isotonic saline were sodium and chloride. To each tube was added as a preservative sufficient merthiolate to give a final concentration of 1/5000. After reweighing, the Thunberg tubes were gassed with a mixture of either 5% carbon dioxide in nitrogen or 5% carbon dioxide in oxygen. The tube containing the sample to be incubated was placed in a 37° C. water bath for periods of time varying from 1 to 14 days. Immediately after gassing, the brain slices in the control tube were removed and extracted with a 1 : 1 ethyl alcohol – ether mixture as outlined previously (Johnson, McNabb, and Rossiter (6)).

The tubes were not regassed during incubation and after 14 days the pH, determined by glass electrode, had not changed. After incubation, a sample of the medium was taken for bacterial culture, the slices removed, and the lipids extracted as described for the controls.

Autolysis of Nerve

Both sciatic nerves of a cat were removed with aseptic precautions and treated in a similar manner to the brain slices. The nerve to be incubated was transferred to a Thunberg tube containing Krebs bicarbonate buffer and gassed with a mixture of 5% carbon dioxide in nitrogen. The tube was placed in a water bath at 37° C. for eight days. An incubation period of eight days was selected for it corresponded to one of the intervals used in the experiments on degeneration of nerves *in vivo* (Johnson *et al.* (8)).

Estimation of Lipids

The methods adopted for the estimation of the lipids were the same as those described previously (Johnson *et al.* (6)). The concentration of cerebroside, free and total cholesterol, total phospholipid, monoaminophosphatide, and lecithin was determined and from these figures was calculated the concentration of ester cholesterol, sphingomyelin, and cephalin. It has been shown that the coefficient of variation of the over-all procedures were as follows: cerebroside ± 3.5%, free cholesterol ± 0.9%, total cholesterol ± 0.9%, total phospholipid ±1.3%, monoaminophosphatide ± 1.6%, lecithin ± 5.1%, sphingomyelin ± 11.9%, and cephalin ± 3.9%. Since the estimations on brain were done in triplicate and those on nerve in duplicate, the probable error of each observation was, therefore, less than the above figure.

Results

The results were recorded as the difference between the concentration of each individual lipid in the control sample and the concentration of the same lipid in the incubated sample, expressed as a percentage of the control. Five or six experiments were done in each group and the mean and standard error of the mean presented for each lipid (Tables I to IV). The variation from experiment to experiment was great. This is brought out in the tables by the high value of the standard error of the mean.

TABLE I

MEAN PERCENTAGE CHANGE IN LIPIDS OF CAT BRAIN, INCUBATED 24 HR. IN CO_2–N_2 MIXTURE (FIVE EXPERIMENTS)

	Bicarbonate buffer with saline			Krebs bicarbonate buffer		
	Mean change (%)	S.E.M.	*P*	Mean change (%)	S.E.M.	*P*
Cerebroside	+ 0.28	± 5.70	>0.9	−10.30	±4.26	>0.05
Free cholesterol	+ 5.38	± 7.35	>0.4	+ 0.72	±2.11	>0.6
Total cholesterol	+ 4.52	± 6.54	>0.5	− 0.10	±3.01	>0.9
Total phospholipid	+ 7.30	± 9.80	>0.5	− 7.50	±4.55	>0.1
Lecithin	−18.70	±15.54	>0.2	+ 2.68	±1.18	>0.05
Sphingomyelin	+ 0.34	±16.24	>0.9	− 1.90	±9.65	>0.8
Cephalin	− 3.02	±10.90	>0.7	−13.10	±4.44	**<0.02**

TABLE II

MEAN PERCENTAGE CHANGE IN LIPIDS OF CAT BRAIN, INCUBATED SEVEN DAYS IN KREBS BICARBONATE BUFFER (FIVE EXPERIMENTS)

	CO_2/N_2			CO_2/O_2		
	Mean change (%)	S.E.M.	*P*	Mean change (%)	S.E.M.	*P*
Cerebroside	+ 4.02	± 8.00	>0.1	− 1.80	±19.05	>0.9
Free cholesterol	− 7.08	± 7.25	>0.2	− 1.40	± 3.51	>0.7
Total cholesterol	− 6.90	± 6.93	>0.3	− 0.30	± 3.97	>0.9
Total phospholipid	−14.38	± 4.74	**<0.05**	−30.30	± 5.44	**<0.01**
Lecithin	+ 6.20	± 7.05	>0.4	−13.26	±12.78	>0.3
Sphingomyelin	− 8.50	±20.95	>0.7	−48.72	±13.54	**<0.01**
Cephalin	−29.82	± 6.45	**<0.02**	−36.24	± 5.32	**<0.01**

There was no significant change in the concentration of lipids of brain slices incubated for 24 hr. in bicarbonate buffer with normal saline (Table I).

TABLE III

MEAN PERCENTAGE CHANGE IN LIPIDS OF CAT BRAIN, INCUBATED 14 DAYS IN KREBS BICARBONATE BUFFER (CO_2–N_2 MIXTURE)

	No. observations	Mean change (%)	S.E.M.	*P*
Cerebroside	5	− 5.16	±18.15	>0.8
Free cholesterol	6	+ 1.38	± 2.65	>0.6
Total cholesterol	6	+ 3.44	± 2.08	>0.1
Total phospholipid	6	−52.66	±10.20	<0.01
Lecithin	4	−18.90	±16.76	>0.3
Sphingomyelin	5	−58.40	± 9.40	<0.01
Cephalin	4	−46.00	±10.28	<0.05

TABLE IV

MEAN PERCENTAGE CHANGE IN LIPIDS OF CAT SCIATIC NERVE, INCUBATED EIGHT DAYS IN KREBS BICARBONATE BUFFER (CO_2–N_2 MIXTURE)

	No. observations	Mean change (%)	S.E.M.	*P*
Cerebroside	5	− 9.58	±11.57	>0.4
Free cholesterol	6	+ 0.08	± 6.13	>0.9
Total cholesterol	6	+ 0.07	± 5.78	>0.9
Total phospholipid	6	−21.20	± 9.10	<0.1
Lecithin	5	−17.10	±15.10	>0.3
Sphingomyelin	5	−14.50	±11.50	>0.2
Cephalin	5	−27.60	± 8.12	<0.05

When Krebs bicarbonate buffer was used, with the exception of cephalin, there was still no significant change in lipid concentration. That there was no significant difference between the percentage change in the total phospholipid concentration when the brain was incubated in bicarbonate buffer with saline and when it was incubated in Krebs bicarbonate buffer confirms the work of Sperry (22) on rat brain homogenates.

A great decrease in total phospholipid, sphingomyelin, and lecithin was observed in brain slices accidentally contaminated with *Clostridium welchii.* These results are interesting, since this microorganism has been shown to hydrolyze lecithin and sphingomyelin (MacFarlane and Knight (13), MacFarlane (12)). Other contaminating organisms did not produce this change. All samples that gave a positive culture were discarded.

There was a significant decrease in total phospholipid, sphingomyelin, and cephalin after incubation for seven days in Krebs buffer in carbon dioxide – oxygen mixture, whereas only total phospholipid and cephalin were decreased significantly when the oxygen of the gas mixture was replaced by the nitrogen

(Table II). However, when the means of the values obtained for each lipid in carbon dioxide and nitrogen were compared with those obtained for the corresponding lipid in carbon dioxide and oxygen, no significant difference in the results was found. The drawing of any conclusion concerning a difference in behavior between slices incubated in oxygen and those incubated in nitrogen is, therefore, questionable.

When autolysis in carbon dioxide and nitrogen was prolonged to 14 days, there was a significant decrease in concentration of total phospholipid, sphingomyelin, and cephalin with no significant change in the concentration of lecithin (Table III). The concentration of cerebroside and cholesterol did not change significantly even after 14 days.

In Fig. 1 is presented the percentage change in the "essential lipids" i.e. cerebroside, total cholesterol, and total phospholipid. Each point is the mean percentage change of all samples incubated for similar periods of time

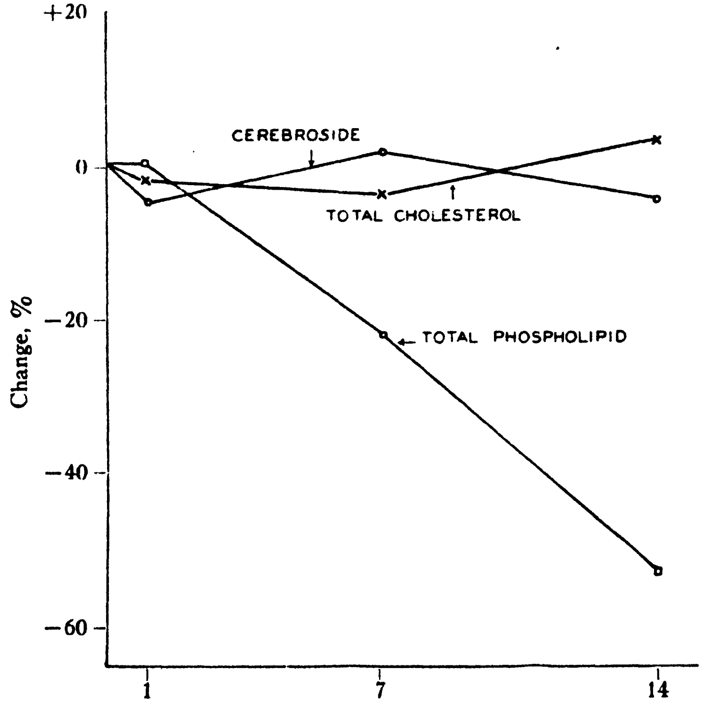

FIG. 1. *The percentage change in 'essential lipids' i.e. cerebroside, total cholesterol, and total phospholipid after incubation in bicarbonate buffer at 37° C. Each point represents the mean of 10 observations except of those for 14 days, which represent the mean of six observations.*

regardless of buffer or gas mixture. It can be seen that, whereas there was a great decrease in total phospholipid, there was no change in cerebroside or total cholesterol. Fig. 2 shows the percentage change of the individual phospholipids. There was a great change in the concentration of sphingomyelin and cephalin and no significant change in the concentration of lecithin.

In sciatic nerve, incubated for eight days in Krebs bicarbonate buffer, there was a significant decrease in cephalin and a decrease of borderline significance in total phospholipid (Table IV). There was no significant change in the concentration of cerebroside, free or total cholesterol, sphingomyelin, or lecithin.

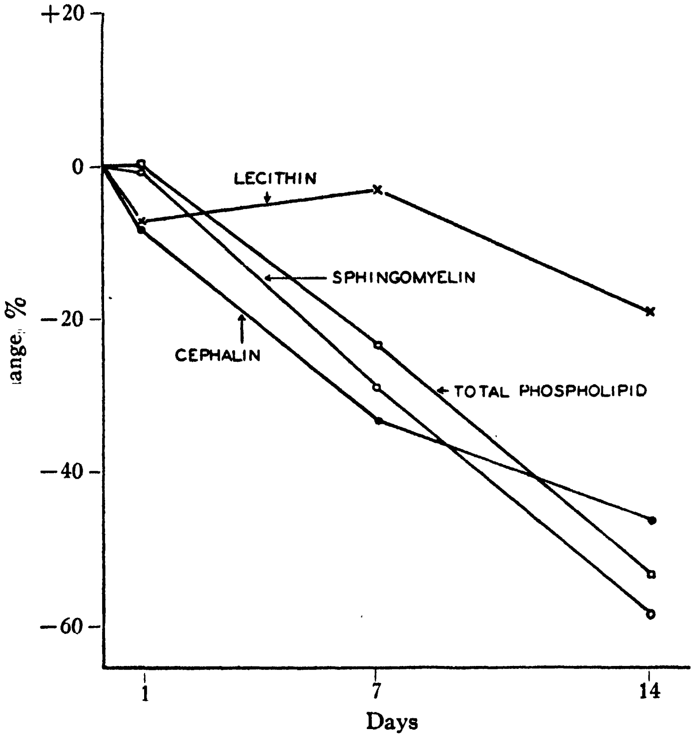

FIG. 2. *The percentage change in phospholipids after incubation in bicarbonate buffer at 37° C. Each point represents the mean of 10 observations except those for 14 days, which represent the mean of six observations.*

Discussion

When a peripheral nerve undergoes Wallerian degeneration there is little change in the concentration of cerebroside, free cholesterol, or sphingomyelin, the substances that we have previously suggested are the principal lipids of the myelin sheath of nerve (6, 7, 9), or in the concentration of cephalin, during the first eight days after nerve section (8). When the nerve had autolyzed for eight days *in vitro*, there was a significant decrease in cephalin, a decrease of borderline significance in total phospholipid, with no significant change in any of the other lipids. Thus it appears that *in vitro* the nerve degenerated more rapidly than *in vivo*. In this connection it is interesting to recall the observation of Cajal (2) that the histological changes characteristic of Wallerian degeneration proceed more rapidly *in vitro* than *in vivo*.

A comparison between the *in vitro* autolysis of brain and the *in vivo* degeneration of nerve, already described (Johnson *et al.* (8)), is of interest. At the end of seven days, the changes in total phospholipid, sphingomyelin, and cephalin in brain were considerably greater than those observed for nerve at the end of eight days. At the end of 14 days, the percentage change in phospholipid was similar. In both cases there was a substantial fall in the concentration of cephalin and sphingomyelin and little change in lecithin. The results for the other lipids were, however, quite different. In nerve degenerating *in vivo*, there was a definite decrease in the concentration of cerebroside and total cholesterol with a great increase in ester cholesterol. It thus appears that the enzyme systems concerned with the degeneration of nerve *in vivo* differ from those concerned with the *in vitro* autolysis of brain tissue. This is perhaps hardly surprising, since, *in vivo*, macrophages appear along the course of the degenerating nerve and contribute their enzymes to the degradation mechanism.

It will be noted that there was little change in the concentration of lecithin in either autolyzing brain tissue or in nerve degenerating *in vivo*. Lecithin was determined as the choline liberated from the mixed phospholipid extract after hydrolysis in *N* potassium hydroxide for 18 hr. at 37° C. It is possible that the autolyzed sample contained unknown degradation products of either lecithin or sphingomyelin that would yield choline on hydrolysis under these conditions. It is unlikely that the lecithin figures are unduly high for this reason, since known intermediates, such as α-glycerylphosphorylcholine studied by Schmidt, Hershman, and Thannhauser (20), are petroleum ether insoluble and would not be found in the phospholipid extracts.

Much of the early work on brain autolysis was concerned with the degradation of proteins rather than of lipids. When the importance of the brain lipids became generally appreciated, reports appeared on the change in lipid content of brain during autolysis. Page (18) advanced a hypothetical scheme for the biochemical changes in lipids during demyelination. This scheme was based, in part, on the evidence of Jungmann and Kimmelstiel (10) and of Backlin (1). The former workers found that cerebroside decreased and inorganic phosphorus increased when rabbit whole brain stood in either oxygen or nitrogen. Backlin (1) described a fall in free cholesterol and cerebroside and a possible increase in phospholipid when a rabbit whole brain stood for 24 hr. However, his figures are the results of only one experiment and are not significant. Sperry, Brand, and Copenhaver (23) found little change in the concentration of total cholesterol and total phospholipid in one half of a rat's brain, incubated in 0.9% sodium chloride for one day. Under the conditions of our study, there was no change in cerebroside or total cholesterol of cat brain slices even after 14 days. Although there was no change in total phospholipid after 24 hr., the decrease following longer periods of incubation has been confirmed. It should be pointed out that many of the above workers did not mention a buffer system and it is likely that the hydrogen ion concentration was not controlled.

Simon (21) found that inorganic phosphorus, which appeared during brain autolysis, was derived from alcohol–ether soluble material, presumably phospholipid, a result confirmed by Stamm (24). Coriat (3) reported that choline was liberated when minced brain was autolyzed for 72 hr. in the presence of chloroform. The possibility that autolyzing brain tissue contains a lecithinase is suggested by the work of King (11), who found that dispersions of rabbit and chicken brain liberated small and variable quantities of free and acid soluble phosphorus from a lecithin emulsion. With homogenates the hydrolysis of phospholipids is apparently much faster. This is shown by the work of Fries *et al.* (5) and Sperry (22) who demonstrated a constant but small decrease in total phospholipid concentration when homogenates of brain tissue were incubated for only four hours.

A possible interpretation of these results that must be considered is that the physical properties of the phospholipids are altered in such a way that they are no longer extracted by the procedures employed. This problem was fully investigated by Sperry (22), who used a number of different extraction procedures and obtained similar results. His results, and our finding that cholesterol and the relatively insoluble cerebroside were completely extracted after incubation, would indicate that hydrolysis of phospholipid does occur.

This work suggests that in tissue of the central nervous system, there is a phospholipid-splitting mechanism, possibly enzymatic in nature. It has not been possible to demonstrate the degradation of either cerebroside or cholesterol.

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SOME FACTORS AFFECTING THE AUSCULTATORY MEASUREMENT OF ARTERIAL BLOOD PRESSURES[1]

By A. E. Thomson and J. Doupe

Abstract

Auscultatory blood pressure measurements have been compared to intra-arterial lateral and end pressures. It was found that auscultatory measurements, which are dependent upon the penetration of pulse waves through a compressed segment of artery, were influenced by various factors. When auscultatory measurements approximated or exceeded intra-arterial pressures, broad pulses were found; when auscultatory measurements were below intra-arterial pressures, narrow pulses were found. By measuring tissue pressures under a cuff it was shown that cuffs subtended only a relatively short narrow band of equal pressure into the tissues. Hence narrow cuffs or, conversely, large arms that allowed only a fraction of the applied pressure to reach the artery caused high auscultatory measurements of both systolic and diastolic pressure. It was concluded that pulse contour and arm size were major causes of the auscultatory systolic errors while the diastolic errors were due to arm size plus unknown factors.

Introduction

It has been pointed out by Ragan and Bordley (2) that auscultatory measurements of blood pressure are frequently in error. In 40 patients the range of deviation of systolic measurements from lateral intra-arterial pressures was − 20 to + 47 mm. Hg, and for diastolic measurements the range was − 12 to + 39 mm. Hg. Their work and that of others (3, 4, 5) indicated that the auscultatory measurements were affected by the contour of the pulse, the girth of the arm, and the width of the blood pressure cuff. The present report is mainly concerned with the mechanism by which these factors produce their effects and is not primarily concerned with the extent of the error nor with the difficulties of interpretation caused by the variability of the pressure.

Method

The values obtained by the standard auscultatory procedure (7) have been compared to intra-arterial pressure measurements obtained by using a variable capacitator similar to that described by Lilly (1). A hollow 20 gauge needle inserted into the brachial artery transmitted the pressure to a phosphor-bronze diaphragm that formed one plate of a condenser in a radio frequency circuit. The position of the diaphragm controlled the frequency and this in turn determined the output of an amplifier that fed an oscillograph galvanometer whose deflections were recorded on moving bromide paper. At approximately five-minute intervals during a recording, the manometric system was calibrated by the application of known pressures to the diaphragm. Measurements approximating lateral pressure were obtained by this method. To obtain end pressures the circulation was occluded by a cuff immediately distal to the needle. The frequency characteristics of the apparatus were

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[1] *Manuscript received November 12, 1948.*

Contribution from the Departments of Medicine and Medical Research of the University of Manitoba and the Winnipeg General Hospital, Winnipeg, Man.

not accurately ascertained but the response time of the system, with attached 20 gauge needle, for a sudden fall in pressure from 200 to 0 mm. Hg, was less than 5 msec. The other methods employed will be described with the results.

Subjects with appropriate arm sizes, who did not exhibit obvious cardiovascular disorders, were selected, and all measurements were taken with the subjects at rest in the recumbent position. In certain cases the circulation to the extremity was increased by general body heating and vigorous exercise of the arm muscles. Arm circumference was measured at a point midway between the acromion and the olecranon. In the subjects in whom the intra-arterial pressures of one arm were compared to the auscultatory measurements of the other arm it was required that the auscultatory measurements of the two arms did not differ by more than 5 mm. Hg.

Results

In common with those of other investigators the present results have shown certain discrepancies between auscultatory and intra-arterial pressure measurements. These will be described first and related as far as possible to the pulse contour, then the influence of the width of the blood pressure cuff, and the girth and consistency of the arm will be considered and finally observations of the tissue pressure will be reported.

Auscultatory and Lateral Pressures

Simultaneous auscultatory and lateral pressure measurements were obtained three to five times in 13 experiments on 10 subjects. As the relationship between the two measurements in each experiment was constant to within 5 mm. Hg, only their averages are recorded in Table I. In these experiments

TABLE I

SIMULTANEOUS AUSCULTATORY AND INTRA-ARTERIAL PRESSURES

(Measured in mm. Hg)

Subject	Arm girth, cm.	Crest time,** msec.	Systolic			Diastolic		
			Ausc.	Lateral	Diff.	Ausc.	Lateral	Diff.
D.C.	25	120	147	157	− 10	79	62	+ 11
E.L.	23	130	120	122	− 2	70	65	+ 5
V.M.	26	160	131	145	− 14	70	70	+ 0
H.J.	23	170	125	126	− 1	80	52	+ 28
H.K.	23	200	102	105	− 3	68	56	+ 12
M.G.	21	200	151	148	+ 3	53	57	− 4
G.T.	26	200	165	162	+ 3	80	59	+ 21
R.F.	27	200	118	120	− 2	78	64	+ 14
P.B.	27	220	124	121	+ 3	79	65	+ 14
V.M.*	26	300	136	131	+ 5	78	56	+ 22
H.K.*	23	300	101	96	+ 5	70	58	+ 12
T.K.	30	330	124	116	+ 8	81	69	+ 12
T.K.*	30	400	122	112	+ 10	81	76	+ 5

* *Subjects in whom blood flow was increased by heat and exercise.*

** *Measurement described in text.*

auscultatory systolic measurements averaged 0.4 mm. Hg above lateral systolic pressure with a range of difference from minus 14 to plus 10 mm. Auscultatory diastolic measurements, using muffling of sounds as an index, averaged 12.4 mm. Hg above lateral diastolic pressure with a range of difference from minus 4 to plus 28 mm. This is in accord with the findings of other investigators (2, 4, 5). The discrepancy between diastolic measurements disappeared if the abrupt cessation of sounds was used as a criterion for the auscultatory measurement as reported by Steele (5), but ordinarily the transition was so gradual that this point could not be detected.

Contour of the Lateral Pressure Pulses

Information as to one of the causes of the observed discrepancies of the systolic measurements was obtained by analyzing the contour of the lateral pressure pulses in the individual cases. The shape of the pulses differed from individual to individual and in the same individual with changes in circulatory state. To obtain an index of their relative peakedness the average duration of three representative waves 15 mm. Hg below the systolic peak was determined. These measurements of "crest time" appear in Table I and the subjects have been arranged so that they progress from those with the most peaked to those with the most rounded pulses. It will be seen that the auscultatory systolic measurements were below lateral pressures when the pulse crest was narrow, approximately the same when the crest was intermediate in type, and above when the crest was broad. No such relationship could be demonstrated for the discrepancies in diastolic measurements. These observations are also in agreement with those reported by others (2, 4).

Difference Between End and Lateral Pressures

Despite the observed relationship of pulse contour to the difference between auscultatory and lateral systolic pressures it seemed unjustified to stress this relationship when theoretical considerations suggested that end and not lateral pressures should be used for the comparison. To test this possibility auscultatory measurements were made before and after end pressures had been produced by inflating a cuff on the forearm to beyond systolic pressure. This was done three times on four subjects both before and after heating and exercise. In no instance did inflation of the cuff produce a change greater than 4 mm. Hg in either the systolic or diastolic measurement. To show that obstruction of the flow did cause a significant change in pressure eight experiments were done on five subjects by inflating a cuff just distal to the site of arterial puncture. For comparison three measurements of pressure were made prior to the inflation of the cuff and three measurements after inflation in approximately the same phase of respiration. The averages of these observations are presented in Table II. In every instance a rise in systolic pressure occurred and, for the group, averaged 9 mm. Hg with a range of 3 to 17 mm. This rise in pressure was accompanied by a change in pulse contour and the extent of the rise was related to the degree of narrowing of the pulse as

TABLE II

END AND LATERAL INTRA-ARTERIAL PRESSURES

(Measured in mm. Hg)

Subject	End pressure		Lateral pressure		Difference (end minus lateral)		% narrowing of pulse**
	Sys.	Dias.	Sys.	Dias.	Sys.	Dias.	
E.L.	140	63	125	61	15	2	33
V.M.	166	68	149	63	17	5	32
H.K.*	109	61	98	59	11	2	33
T.K.*	121	81	113	79	8	2	30
T.K.	117	73	112	70	5	3	25
V.M.*	119	68	113	67	6	1	13
H.K.	116	59	113	56	3	3	10
M.G.	149	58	146	57	3	1	0

* *Subjects in whom blood flow was increased by heat and exercise.*
** *Calculated from Crest Times in Tables I and III.*

shown in Table II. As in the case of auscultatory diastolic measurements no significant change was produced in intra-arterial diastolic pressure by obstructing the flow. These observations agree in general with those of Robinow *et al.* (4).

Because systolic end pressures differed materially from lateral pressures and because auscultatory systolic measurements were not affected by obstruction of the blood flow, it was apparent that auscultatory measurements are made when end pressures exist and therefore should be compared to them.

Auscultatory Measurement and End Pressures

The comparison between systolic auscultatory and end pressures has been made in Table III utilizing data from Tables I and II. It will be seen, owing to the alterations produced in the pulse wave contour and in the systolic pressure by obstructing the flow, that the discrepancies between auscultatory and intra-arterial pressures have been altered. The net effect of changing

TABLE III

DISCREPANCY BETWEEN AUSCULTATORY AND END SYSTOLIC PRESSURES

Subject	E.L.	V.M.	H.K.	M.G.	H.K.*	T.K.*	T.K.	V.M.*
End crest time (msec.)	80	100	180	200	200	230	250	260
Ausc. minus end (mm. Hg)	−17	−31	−6	0	−6	−2	−3	−1

* *Subjects in whom blood flow was increased by heat and exercise.*

the standard of comparison from lateral to end pressures has been to accentuate the differences associated with narrow pulses and to minimize those associated with broad pulses.

Influence of Cuff Width and Arm Girth

To show the influence of cuff width and arm girth on auscultatory measurements, a group of subjects was chosen in which there was a much wider variation in regard to arm circumference than in the group just considered. On 18 subjects, whose arm circumference ranged from 20 to 38 cm., three auscultatory measurements of systolic and diastolic pressure were made with each of four cuffs, 8, 12, 16, and 20 cm. in width. The group was divided into three equal classes, those with arm circumferences from 20 to 26 cm., from 26 to 32 cm., and over 32 cm. The mean values of each class for the four widths of cuff are plotted in Fig. 1, which shows that the highest measurements of both systolic and diastolic pressure were obtained using the 8 cm. cuff and the greater the girth of arm the greater the effect of changing the width of the cuff, the initial slope of the curves being steepest in the class with the greatest arm girths.

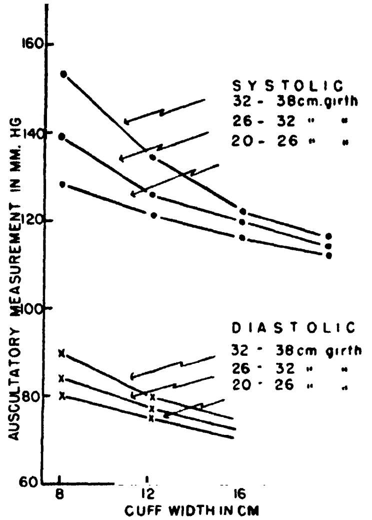

FIG. 1. *Effect of cuff width on auscultatory measurements in subjects with different arm girths.*

As the above results indicated the importance of arm girth, an attempt was made to ascertain the relationship of measured girth to effective girth, i.e., the girth after inflation of the cuff. To determine this, using a soft tissue technique, X-ray films were taken before and after inflation of a standard blood pressure cuff to systolic pressure. The measured girths of three subjects whose soft tissues ranged from firm to flabby were 29, 30, and 34 cm. Girths calculated from X-ray films before inflation were 29, 30, and 35 cm. and after inflation 27, 26, and 30 cm. Clearly effective girth differed from measured girth and this difference appeared to depend upon the consistency of the soft tissues.

Distribution of Pressure in the Tissues Under a Cuff

The pressure in the tissues under a blood pressure cuff was measured using a modification of the apparatus described by Wells *et al.* (6) in which the water in the measuring column was replaced by mercury. Measurements were made using four or more cuff pressures between 20 and 200 mm. Hg at four points under two cuffs of different widths. Those for the 12 cm. cuff are plotted in Fig. 2 and show that for each point in the tissues the ratio of tissue pressure to applied pressure was constant. The constancy of this ratio indicated that the spatial distribution of the relative pressure beneath a cuff was independent of the level of the cuff pressure.

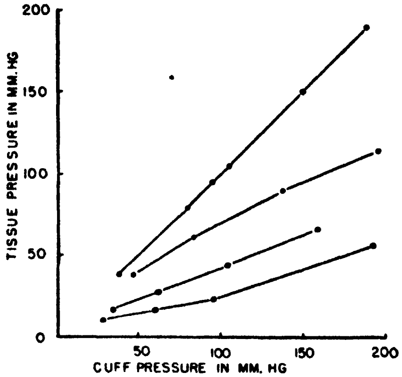

Fig. 2. *Effect of varying the cuff pressure on the pressure at four positions in the tissues.*

The distribution of pressure was ascertained on one individual by measuring the tissue pressure at 29 different points under a 12 cm. cuff, nine under an 8 cm. cuff, and 26 under a 4 cm. cuff. The position of these points was estimated by the depth of insertion and the angle of the needle and was probably accurate to within 0.5 cm. in any direction. All the measurements were converted into percentage values of the cuff pressure and were located in the diagram shown in Fig. 3, keeping constant their relationship to the nearest edge of the cuff and their depth in the tissues. Because the discrepancies between the measurements made with different cuffs were no greater than those between the measurements made with the same cuff, it was concluded that the pattern of the pressure distribution was similar for the various cuffs. The total number of observations was insufficient to enable exact isobaric lines to be drawn, but a general pattern was obvious. The highest relative pressures were found near the center of the cuff, and the zone occupied by these values, although fairly extensive immediately beneath the cuff, became less so at greater depths in the tissues. With the 4 cm. cuff the 100% zone was not detected. Lowest relative pressures were found towards the edge of the cuff and the area occupied by these values was definitely greater at increasing depths in the tissues. The zone of intermediate values appeared to broaden at greater depths in the tissues at the expense of the high pressure zone.

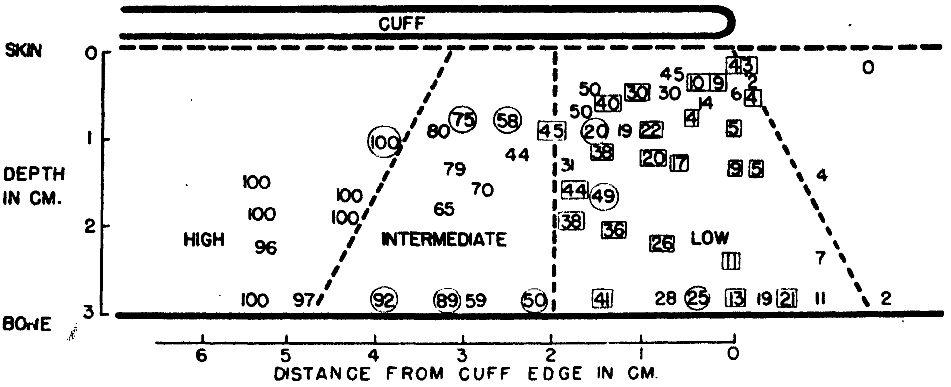

FIG. 3. *Diagrammatic longitudinal section of arm showing distribution of pressure in the tissues under a cuff. Pressures are expressed as a percentage of cuff pressures. Those within squares and circles and the others were obtained with 4, 8, and 12 cm. cuffs, respectively. Borders of zones are indicated by dotted lines.*

The application of the above results to the present problem was shown by making auscultatory measurements on the same patient with different widths of cuffs. These are shown in Fig. 4 together with estimates of the

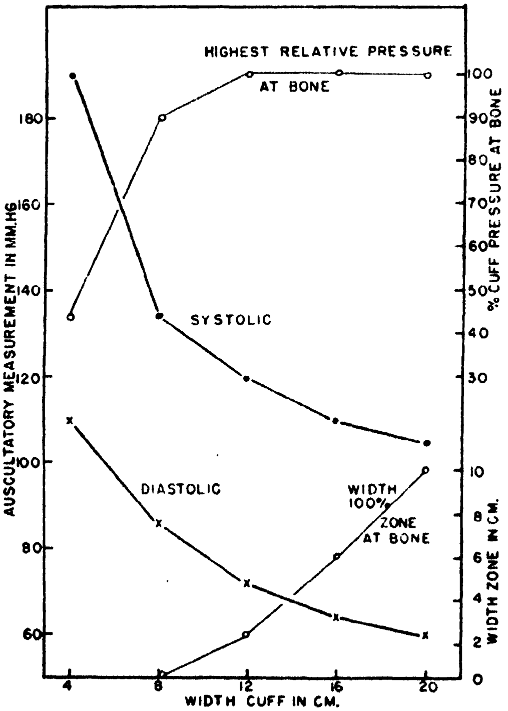

FIG. 4. *Effect of cuff width on auscultatory measurements compared to estimates of relative tissue pressure at the level of bone.*

relative pressure at the level of bone taken from Fig. 3. The steep parts of the systolic and diastolic curves appear to correspond to the transition from a cuff that did not project a high relative pressure zone as far as the bone to one that did. The less steep parts of the curves correspond to increases in width of the high pressure zone produced by changing to still wider cuffs.

Discussion and Summary

The present results indicate some of the reasons why auscultatory measurements are inaccurate. It was first shown that auscultatory systolic measurements were influenced by the shape of the pulse wave even after taking into consideration the phenomena associated with the conversion of lateral to end pressures. The explanation of this relationship seems obvious. The production of audible sounds depends upon the penetration of the peaks of the pulse waves through the segment of artery compressed by the cuff. A certain amount of energy is necessary to overcome the pressure exerted by the cuff. A pulse that is broad and rounded will have considerably more energy available at its peak to penetrate the compressed segment than will a narrow pulse. Thus when auscultatory systolic measurements approximated or exceeded intra-arterial pressures broad pulses were found; when auscultatory measurements were below intra-arterial pressures, narrow pulses were found. This theory also affords an explanation for the lack of relationship between the diastolic errors and the shape of the pulse waves.

It was then shown in a series of experiments, which were essentially a repetition of those of von Recklinghausen (3), that the use of narrow cuffs gave high systolic and diastolic auscultatory readings. This would be expected because it may be presumed that a narrow segment of compression will offer less resistance to a pulse wave than will a wide one, necessitating a higher compression pressure on a narrow segment to produce an equivalent resistance. However, the results also showed that this phenomenon was not similar in all ranges of arm girth. In the larger arms, narrow cuffs required a much higher pressure to obstruct the pulse than was expected. The observations of the tissue pressure under various cuffs showing that only a fraction of the applied pressure was being transmitted to the artery provided an explanation for these findings.

It was also shown that the consistency of the tissues affected the distortion of the arm caused by inflating a blood pressure cuff. This would make it difficult to deduce the effective girth from measurements of the uncompressed arm. It would appear likely, although the point was not investigated, that the consistency would also affect the distribution of pressure in the tissues.

The application of these results to clinical work merits attention. At the present time it may be justifiable on practical grounds to ignore the effect of pulse contour on systolic measurements because the significance of the more transient peaks of pressure is not known. The auscultatory method would even have an advantage, if it could be shown that such transient peaks were so low in energy as not to materially affect either the walls of the blood vessels or the rate of flow. However, it is not justifiable to disregard the effect of arm girth particularly as this also modifies the diastolic measurement. Finally it should be stressed that the cause for many of the diastolic discrepancies has evaded analysis.

Acknowledgments

This study was performed while one of us, A.E.T., held a Fellowship of the Life Insurance Research Fund. It was supported by a grant from the National Research Council of Canada and by a gift for equipment from Mr. W. McG. Rait (Winnipeg). The authors are indebted to Dr. John McEachern for advice and to Mr. T. W. Acheson for technical assistance.

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EFFECT OF AMYTAL ON SKELETAL MUSCLE[1]

By Bernard E. Riedel and Mervyn J. Huston

Abstract

Sodium amytal, 190 mgm. per kgm., injected intraperitoneally into rats caused a transient increase in response of normal and denervated striated muscle to electrical stimulus. That this effect is not due to changes in pH nor to changes in ionic balance has been shown by pH tests on rat blood and by intraperitoneal administration of solutions of sodium hydroxide. It is believed that the increased response is due, at least in part, to a direct action on the muscle.

Introduction

Gross and Cullen (4) have shown that sodium pentothal in very high blood concentration depresses the response of striated muscle to intra-arterial injections of acetylcholine. A depressant effect of pentobarbital on the knee jerk and flexion reflex was reported by Van Harreveld (18). Huston, Martin, and Dille (11) demonstrated that pentobarbital and seconal depress the somatic neuromyal junction at concentrations 10 to 20 times the estimated effective concentrations at other synapses. At this concentration, however, an initial stimulant effect on the muscle was noted. It was thought of interest to examine further the action of barbiturates on the somatic system. This paper will deal with the results obtained with sodium amytal on the rat.

Method

The spinal cord of the rat was sectioned at the upper lumbar region under ether anesthesia and the rat left for 24 hr. to recover from the effects of the anesthetic. The animal was then tied down on its back on a small table and one hind leg was securely clamped at the knee and ankle. The calcaneous tendon was freed from the skin and fascia and severed at its attachment to the calcaneous bone. Recording was by means of an isometric spring attached to the calcaneous tendon.

The sciatic nerve was cut and the peripheral stump clamped in a shielded electrode. When denervated preparations were used, stimulation was through leads stitched in at each end of the gastrocnemius muscle. Stimulus was provided by an electronic stimulator at 10-sec. intervals. A maximal stimulus was used throughout.

A normal level of response to stimulation was obtained for approximately 90 min. before administration of the drug. The measurement of response

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[1] *Manuscript received December 17, 1948.*

Contribution from the School of Pharmacy, University of Alberta, Edmonton, Alta. Presented at the First Pan-American Congress of Pharmacy, held in Havana, Cuba, December 1-8, 1948.

was made from the resting level of the muscle and was calculated as force of contraction in grams on a calibrated isometric spring. Experimental changes were reported as a percentage of the contraction tension before injection.

Results

Since the response of the normal muscle was found to decrease slowly, it was necessary to run a series of control animals to establish a criterion for the experimental results. Table I presents the results obtained with 20 normal rats. It will be noted that the mean shows a slow decline in response.

TABLE I

RESPONSE OF NORMAL GASTROCNEMIUS MUSCLE OF THE RAT TO ELECTRICAL STIMULUS AT 10 SEC. INTERVALS

Figures represent percentage of initial contraction tension

Time in minutes								
3	6	9	12	15	30	45	60	72
100.0	100.0	100.0	100.0	100.0	101.4	101.4	101.4	103.6
97.2	97.2	97.2	97.2	94.5	92.6	—	—	81.9
100.0	100.0	100.0	100.0	98.2	95.3	86.7	80.4	80.4
100.0	95.1	95.1	95.1	91.6	91.6	89.6	84.5	84.5
100.0	100.0	97.3	97.3	96.2	96.2	92.4	88.8	87.0
100.0	100.0	101.0	101.0	101.0	101.0	101.0	101.0	101.0
100.0	100.0	99.0	99.0	99.0	98.2	99.2	99.2	99.2
100.0	100.0	100.0	100.0	100.0	95.4	90.3	88.9	84.2
99.0	99.0	99.0	99.0	99.0	99.0	97.6	96.8	94.4
102.1	102.1	99.4	99.4	99.4	98.6	97.3	97.3	97.3
97.3	97.3	97.3	97.3	93.4	97.3	94.5	92.3	89.6
102.4	100.0	102.4	108.8	108.8	106.4	102.4	102.4	102.4
98.0	93.5	89.0	87.5	85.0	82.0	74.5	76.0	71.5
99.0	104.9	95.1	90.7	88.3	81.9	81.9	84.4	77.6
94.5	94.5	88.3	91.0	88.3	89.6	100.0	94.5	88.3
101.0	104.6	107.2	103.1	101.0	107.2	107.2	108.2	103.1
98.7	98.7	101.7	97.8	93.4	98.7	98.3	95.2	93.9
100.0	101.9	100.0	100.0	96.6	98.1	91.2	91.9	91.9
100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
98.6	98.6	101.0	101.0	101.0	94.8	91.0	91.0	91.0
Mean 99.4	99.4	98.5	98.3	96.7	96.3	94.6	93.4	91.1
S.d.m. 0.39	0.65	0.96	1.02	1.22	1.44	1.79	1.86	2.04

The results obtained with 20 experiments when 190 mgm. per kgm. of sodium amytal was injected intraperitoneally are recorded in Table II. In every case there was an increased response followed by a progressive decrease. In 14 of 20 cases a maximal had been reached within 12 min. after the injection. The maximal mean increase was 13.7%. The highest single increase was 69.2%, which was not reached until 30 min. after the administration of the drug (No. V, Table II). As the dosage level used was very high for rats (14, 15, 16) a number of the animals died within a short time. No readings were taken after respiration ceased.

TABLE II

EFFECT OF SODIUM AMYTAL, 190 MGM. PER KGM., ON THE RESPONSE OF THE NORMAL GASTROCNEMIUS MUSCLE OF THE RAT

Figures represent percentage of initial contraction tension

Time in minutes								
3	6	9	12	15	30	45	60	72
110.0	112.0	110.0	95.0	79.0	—	—	—	—
102.2	111.2	114.6	116.8	119.1	125.8	119.1	114.6	112.4
100.7	102.8	102.8	105.6	101.4	106.3	106.3	106.9	104.9
103.6	105.4	103.6	103.6	100.0	73.2	66.1	65.2	60.7
133.8	153.8	161.5	166.2	161.5	169.2	156.9	143.1	130.8
104.8	109.7	112.7	106.1	95.8	86.1	61.8	71.5	77.6
103.6	108.8	103.6	107.3	106.6	91.2	92.7	92.7	89.1
109.6	112.3	116.4	119.2	112.3	—	—	—	—
100.0	106.0	97.3	96.7	96.0	—	—	—	—
108.6	113.3	113.3	115.2	113.3	—	—	—	—
118.1	137.3	137.3	137.3	112.1	100.0	96.4	—	—
102.8	107.4	110.2	114.8	112.0	112.0	112.0	114.8	120.4
100.0	113.0	113.0	121.0	123.0	124.0	—	—	—
106.0	115.9	121.9	115.9	112.2	106.1	102.4	—	—
104.8	114.3	117.1	112.4	107.6	—	—	—	—
100.0	106.4	106.4	112.8	93.6	76.9	69.2	74.4	74.4
102.8	102.8	107.6	107.6	107.6	—	—	—	—
102.0	120.4	108.2	100.0	87.8	67.4	51.0	32.7	32.7
104.5	104.5	110.7	114.3	119.6	119.6	107.1	89.3	58.9
100.7	102.6	103.7	105.9	108.1	99.3	—	—	—
Mean 105.9	112.9	113.6	113.7	108.4	104.1	95.1	90.5	86.2
S.d.m. 1.76	2.77	3.15	3.46	3.70	7.10	8.43	10.15	9.80

In Fig. 1 a comparison of the normal mean (Table I) and the experimental mean (Table II) is presented graphically. It is apparent that there is a significant initial increase in response in the experimental legs but that the subsequent depression is not significantly different from the control.

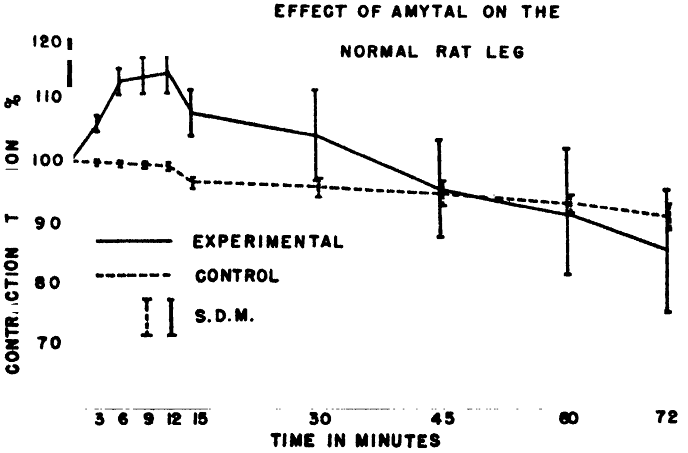

FIG. 1.

It was considered possible that the stimulant effect might be due to the alkalinity of the solution or to the sodium ion. Comparisons were made by means of a glass electrode of the pH of blood from normal and injected rats. No significant difference was noted. The buffers of the blood can apparently compensate for the amount of sodium amytal used. To establish that pH and ionic factors are not involved in the increased response, a series of experiments was run in which sodium hydroxide solution was injected intraperitoneally. The dose of sodium hydroxide used contained a weight of sodium equivalent to the weight of sodium in an experimental dose of sodium amytal. The pH of the sodium hydroxide solution was 10.8. This is higher than the pH of sodium amytal solution, which was 9.9. Table III presents the results obtained with seven such experiments. It is apparent from Fig. 2, where the mean of the normal legs is compared graphically to the mean of the legs treated with sodium hydroxide, that there is no significant difference. It is therefore believed that pH and ionic effects are not responsible for the stimulation.

TABLE III

EFFECT OF SODIUM HYDROXIDE ON THE RESPONSE OF THE NORMAL GASTROCNEMIUS MUSCLE OF THE RAT

(Sodium equivalent to that of sodium amytal 190 mgm. per kgm.)

Figures represent percentage of initial contraction tension

Time in minutes								
3	6	9	12	15	30	45	60	72
97.4	97.4	97.4	97.4	99.1	99.1	99.1	99.1	99.1
100.0	97.3	97.3	100.0	100.0	118.3	118.3	118.3	118.3
100.0	100.0	100.0	100.0	103.0	90.0	78.0	75.0	75.0
100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.5
100.0	97.6	97.6	97.6	97.6	96.0	83.0	68.5	66.6
100.0	100.0	96.3	93.9	93.0	90.7	86.5	82.8	76.2
100.0	100.0	100.0	98.4	96.0	94.4	89.4	89.4	89.4
Mean 99.6	98.9	98.3	98.2	98.4	98.4	93.5	90.4	88.9
S.d.m. 0.37	0.48	0.55	0.83	1.22	3.61	5.11	6.40	6.70

Denervation Experiments

In an attempt to localize the stimulating action, we performed a series of experiments with sodium amytal upon denervated muscles.

The animals were prepared by aseptic section of the sciatic nerve in one thigh. After a period of five days to allow for nerve degeneration, these limbs were studied by the method described above.

Since the decrease of the denervated muscle might be expected to differ from that of nondenervated muscle, a series was run as a control. The results with 12 such rats are tabulated in Table IV. It will be noted by comparison with Table I that the decrease with denervated muscle is slightly more rapid than with normal muscle.

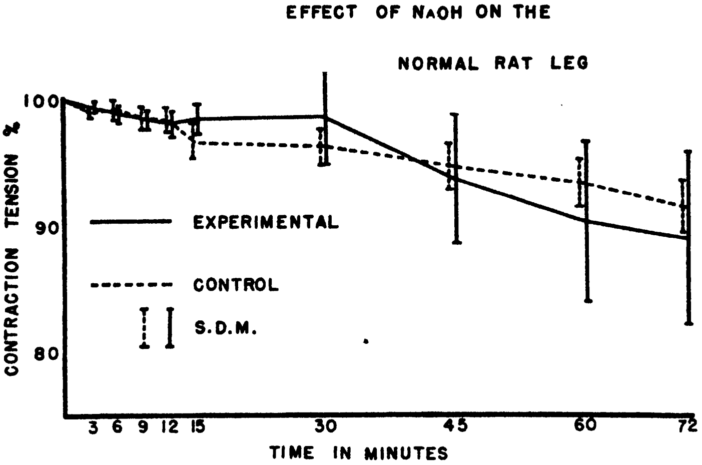

FIG. 2.

TABLE IV

RESPONSE OF DENERVATED GASTROCNEMIUS MUSCLE OF THE RAT TO ELECTRICAL STIMULUS AT 10 SEC. INTERVALS

Figures represent percentage of initial contraction tension

Time in minutes								
3	6	9	12	15	30	45	60	72
97.6	96.1	92.9	88.2	84.3	74.2	74.2	68.7	65.1
97.6	94.3	94.3	93.2	91.0	85.4	82.0	78.6	78.6
98.1	93.5	91.7	91.7	91.7	77.4	68.8	63.3	57.8
100.9	100.9	100.9	96.0	96.0	100.9	93.1	99.0	81.3
96.3	94.4	94.4	94.4	91.6	87.9	87.0	83.3	83.3
100.0	100.0	100.0	100.0	100.0	98.4	96.9	94.5	94.5
100.0	95.0	95.0	92.6	90.1	89.3	89.3	86.8	86.8
100.0	100.0	97.7	97.7	97.7	97.7	95.4	96.9	96.9
100.0	100.0	100.0	100.0	100.0	97.5	97.5	97.5	97.5
100.0	100.0	100.0	100.0	99.1	99.1	98.3	96.0	96.0
97.5	93.0	88.6	87.1	84.6	81.6	74.1	75.6	71.1
99.4	97.3	94.5	96.2	93.4	87.9	83.1	83.1	83.1
Mean 99.0	97.0	95.8	94.8	93.3	89.8	86.6	85.3	82.7
S.d.m. 0.41	0.86	1.11	1.25	1.57	2.57	2.91	3.42	3.69

Table V presents the results obtained when 190 mgm. per kgm. of sodium amytal is injected into rats with denervated legs. The pattern of events is similar to that obtained with normal rats. There is an increase of response

TABLE V

EFFECT OF SODIUM AMYTAL, 190 MGM. PER KGM., ON THE RESPONSE OF THE DENERVATED GASTROCNEMIUS MUSCLE OF THE RAT

Figures represent percentage of initial contraction tension

	Time in minutes								
	3	6	9	12	15	30	45	60	72
	105.0	111.7	106.7	105.8	103.3	84.2	70.8	—	—
	100.9	108.2	102.7	98.2	93.6	75.5	80.0	81.8	—
	103.4	105.7	105.7	109.1	109.1	100.0	71.6	—	—
	103.3	105.6	—	108.9	108.9	103.3	106.7	103.3	103.3
	91.6	100.0	105.9	105.9	110.1	107.6	109.2	103.4	100.8
	102.4	109.4	109.4	103.5	97.7	74.2	82.4	88.2	88.2
	101.2	109.4	109.4	108.2	105.9	97.7	95.3	91.8	88.2
	100.0	102.4	105.9	108.2	108.2	90.6	—	—	—
	101.8	109.2	109.2	109.2	108.3	103.7	—	—	—
	100.0	103.8	102.3	100.0	98.5	82.7	—	—	—
	110.0	107.1	102.9	102.9	100.0	58.3	—	—	—
	103.5	101.2	97.7	90.7	90.7	59.3	—	—	—
	100.0	99.2	92.4	87.0	80.9	60.3	60.3	58.8	58.8
	102.1	104.5	104.5	101.7	98.8	86.4	82.3	75.7	71.6
	107.5	105.4	102.7	101.6	123.5	—	—	—	—
	100.0	99.0	95.0	92.5	88.0	75.0	68.5	64.0	59.5
	105.4	101.4	97.3	84.4	78.2	—	—	—	—
	100.0	102.6	105.3	100.0	100.0	72.8	—	—	—
	101.9	104.7	104.7	99.5	—	—	—	—	—
	103.3	101.1	90.1	78.0	73.6	29.7	17.6	16.5	16.5
Mean	102.2	104.6	102.6	99.7	98.8	80.1	76.8	75.9	73.4
S.d.m.	0.80	0.82	1.30	1.90	2.84	4.26	7.60	9.07	13.75

that reaches a mean peak of 4.6% in six minutes, followed by a decrease. Fig. 3 compares the contraction tension of the denervated experimental legs with those of the denervated control group.

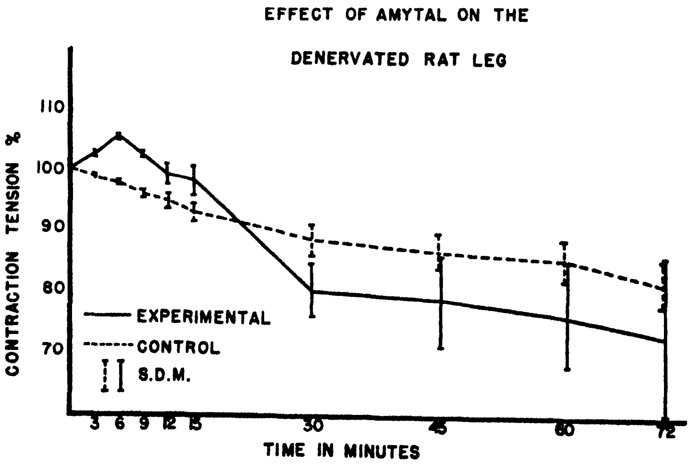

FIG. 3.

Although the increase with the denervated legs is less than with the normal legs this may be due to the more rapid fatigue of the denervated muscle. Table VI presents a comparison of the effects of amytal on normal and denervated muscle when calculation is based on the control curves. There is a peak increase of 15.7% at 12 min. with normal muscle as compared with a peak of 7.8% at six minutes with denervated muscle. Fig. 4 compares the effect on normal and denervated muscle graphically. It would appear that at least part of the effect of amytal is attributable to a direct action on muscle. However, the increased response with denervated muscle was only about half that with normal muscle. This does not necessarily mean that only half the effect on normal muscle is due to a direct action. Denervation is known to cause profound changes in muscle (13, 17) so that the lower response of denervated muscle may be due to the fact that the denervated muscle is less robust and therefore less able to respond to a stimulant.

TABLE VI

COMPARISON OF EFFECT OF SODIUM AMYTAL, 190 MGM. PER KGM., ON NORMAL AND DENERVATED GASTROCNEMIUS MUSCLE OF THE RAT

Figures represent experimental mean as percentage of corresponding control mean

	Time in minutes								
	3	6	9	12	15	30	45	60	72
Normal muscle	106.6	113.6	115.3	115.7	112.1	108.1	100.5	96.9	94.6
Denervated muscle	103.2	107.8	107.1	105.2	105.9	89.2	88.7	88.9	88.7

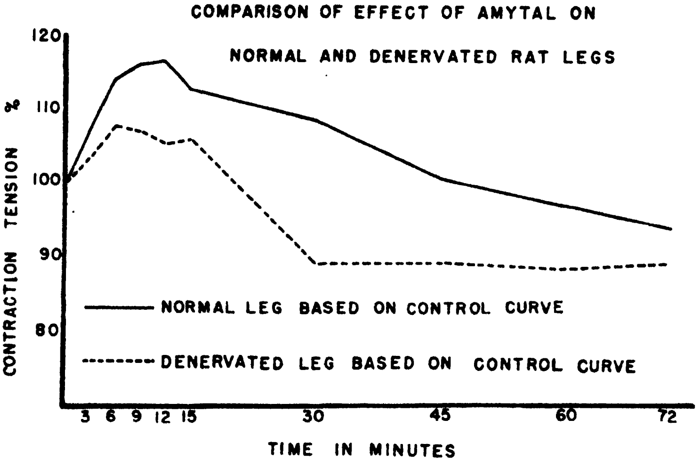

FIG. 4.

Discussion

Huston, Martin, and Dille (11) reported a stimulant effect of pentobarbital and seconal on striated dog muscle, which preceded a depressant effect shown to be on the neuromyal junction. They attributed the stimulant effect to a direct action on the muscle.

Our experiments with sodium amytal therefore agree with the increased response they obtained with pentobarbital and seconal. We were unable to demonstrate any significant depressant action on the neuromyal junction. This is probably due to the fact that we were unable to reach a sufficiently high concentration in the intact animal without causing death by central depression. Huston *et al.* obtained concentrations in an isolated perfused leg that were much higher. The degree of increase noted by us is much less than that reported by Huston *et al.* This may be due to our lower concentration but might also be due to a quantitative difference between amytal, pentobarbital, and seconal. It is known that barbiturates differ quantitatively in their effects on the same part of the nervous system. Such differences on the central nervous system are well known but also have been reported at peripheral points (9). Similar differences might exist in effect on muscle.

The depressant effect that sodium pentothal has on the response of striated muscle to intra-arterial injection of acetylcholine (4), and the depressant effect that pentobarbital and seconal have on the neuromyal junction (11), may be related to the decrease in response of the heart to vagus stimulation under the influence of high dosages of pentobarbital, amytal, and evipal (9), and to the depression of the motility of the gastrointestinal tract by barbiturates (2, 6, 7, 8). There is, however, considerable difference in the sensitivity of different parts of the nervous system to barbiturates (11).

On the other hand a stimulant action of barbiturates on smooth and cardiac muscle has not been reported. This may be due to a difference in response of different types of muscle or it may be that slight or transient effects have been overlooked. Gruber *et al.* (10) in experiments on excised smooth muscle noted a stimulating action with barbiturates but attributed this to the alkalinity of the solution since the effect was not apparent after control of the pH.

A slight decrease in muscle tone was noted after injection of amytal. As calculations are made from the resting or tone level this contributes to the figures recorded. The decrease in tone was slightly more marked in the case of denervated muscle. A similar depression of the tone of smooth muscle by barbiturates has been noted. Gruber and his co-workers have reported a marked decrease in tonus of longitudinal muscle of the intestine (5); a decrease in tonus of stomach muscle (8); and a decrease in general tonus of intact intestine in the unanesthetized dog (2, 6).

It is interesting to note in the case of striated muscle that a decreased tone occurs at the same time as an increased degree of contraction to stimulus.

That the two phenomena are not intimately linked is seen from the fact that the tonus level remains essentially the same after the brief period of stimulation is over.

Denervation of muscle results in a sensitization to acetylcholine (1, 12, 17), which is believed by Cannon and Rosenbleuth (3) to be one example of the general condition of the muscle. Sensitization to barbiturates has not been demonstrated. We believe our experiments to be free from the effect of sensitization for the reasons previously stated (11) and because the stimulant effect of amytal was less with denervated muscle than with normal muscle. Our experiments with amytal would therefore lend support to the contention that sensitization was not of significance in the experiments with pentobarbital and seconal (11).

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THE GASTRIC RESPONSE OF MAN TO AN ACID TEST MEAL[1]

By Gordon E. Bermak and J. Doupe

Abstract

The gastric secretory response to the instillation of 300 ml. of 0.5% hydrochloric acid was investigated 30 times in eight subjects by withdrawing six samples at 20 min. intervals and measuring the acid concentration. In six experiments duodenal regurgitation was prevented by constant duodenal suction. As this did not delay the fall in acidity ordinarily observed it was concluded that regurgitation was not essential for this process. In 14 experiments phenol red was added to the instilled solution and the amount of dilution was measured. It was found that on the average the decrease in concentration of the dye was the same as that of the acid although there were frequent small discrepancies. It was concluded that instillation of acid into the human stomach stimulates one or more secretions whose net effect is to reduce the acidity by dilution.

Introduction

It is obvious that following a rise of gastric acidity some process must take place to restore the acidity to the resting level. At one time it was generally assumed that duodenal regurgitation was the major factor but in 1924 Baird *et al.* (4) demonstrated, by aspirating the duodenal contents, that regurgitation was not essential to the process. In 1928 MacLean and Griffiths (10) presented further evidence indicating that in the human a diluting fluid of neutral character is secreted by the stomach and is chiefly responsible for the reduction of acidity. This denial of the importance of duodenal regurgitation prompted Apperly to review a former study (1) concerning the fate of an acid solution placed in the stomach. He and Norris (3), using an acid solution containing an indifferent substance to serve as an index of dilution, obtained results that were interpreted to mean that neutralization as well as dilution usually takes place and that the neutralizing fluid originates in the duodenum. Nevertheless, the concept of a purely gastric mechanism has continued to obtain support (7, 9, 13). For this reason and because of the potential usefulness of the acid instillation procedure for the investigation of the secretory functions of the stomach (2, 6, 8) it appeared worthwhile to repeat some of the experiments of Apperly and Norris in combination with the aspiration procedure of Baird *et al.*

Method

The acid reduction test of Elman (5) was used. It consisted of aspirating the fasting gastric contents, instilling 300 ml. of 0.5% hydrochloric acid, and withdrawing 20 ml. samples for analysis at 20 min. intervals for 100 min. Precautions against the swallowing of saliva were taken and the gastric contents were mixed by a pumping action of the syringe before each sample was removed.

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[1] *Manuscript received December 14, 1948.*

Contribution from the Department of Medical Research, University of Manitoba, Winnipeg, Man.

In some instances the duodenum was drained throughout the test by the use of a double tube, one lumen of which connected the duodenum to a collecting flask at a pressure of minus 20 cm. water. The tube was considered to be in the duodenum as soon as an alkaline clear amber fluid was withdrawn. The maintenance of this position was confirmed by the continuous flow of a clear, heavily bile stained fluid from the tube varying in amount in different experiments from 350 to 450 ml. In about half the experiments 6 mgm. of phenol red was added to the acid solution. Penner *et al.* (11) have shown that for some purposes the dye method is invalid but in the present experiments, which are not concerned with water absorption, it appears applicable.

Samples were analyzed for free acidity by titrating with 0.1 *N* sodium hydroxide using Topfer's reagent. The concentration of the phenol red was measured with an Evelyn colorimeter using the method of Shay *et al.* (12). In some experiments, particularly those with duodenal drainage, the stomach was empty at 60 or 80 min. so the later samples could not be obtained.

The subjects consisted of eight healthy laboratory workers ranging in age from 19 to 40 years.

Results

The results are presented in Table I. The 12 experiments in which the simple test procedure was alone performed have been placed together in Group *A*. Bile was noted in most of the samples. Group *B* contains the experiments in which phenol red was added to the test solution. The color of the dye obscured bile if it was present. Groups *C* and *D* contain the experiments in which duodenal drainage was performed and differ from each other only in that phenol red was used in Group *D*. No bile was seen in the samples of these groups. Inspection of the data and the average values indicates that the decline in acidity was essentially similar in all the groups. Also presented in the table are the results concerning the degree of dilution undergone by the test solution. For comparison with the acid values, the data pertaining to dilution have been expressed as the normality of acid that would have resulted had the change in concentration of the dye been caused by the addition of a neutral unbuffered solution. To avoid the distortion likely to be introduced by any remaining fasting contents, the calculations were based on the equivalence of the acid and dye concentration in the initial sample rather than in that of the instilled solution. It will be seen that the observed acid values corresponded closely with the calculated values but that there were many minor discrepancies in both directions.

Discussion

If one disregards the possibility that acid may be absorbed in the stomach these results should be applicable to the problem of the site of origin and the nature of the secretion acting on the instilled acid. In regard to the first, the method of preventing regurgitation of duodenal contents would be entirely acceptable only if it did not alter other factors. That they were altered in

TABLE I

CHANGE IN NORMALITY OF AN ACID SOLUTION PLACED IN THE STOMACH

(Figures in parentheses show difference between the measured decline in normality and that predicted from dye dilution measurements. Plus values suggest an alkaline secretion)

Exptl. group	Subject	Acidity initial sample, normality HCl (× 1000)	Decrease in normality from value of initial sample (× 1000)				
			20 min. sample	40 min. sample	60 min. sample	80 min. sample	100 min. sample
A Acid alone	Mr. B.	115	5	20	32	48	55
	Mr. B.	116	18	24	32	43	81
	Mr. B.	124	12	24	35	49	73
	Mr. B.	109	3	9	19	30	53
	Mr. B.	122	10	21	43	66	67
	Mr. D.	115	16	27	60	67	70
	Mr. D.	126	11	26	35	52	64
	Mr. D.	130	16	30	46	55	69
	Mr. D.	132	19	23	52	75	82
	Mr. D.	126	6	18	29	66	79
	Mr. D.	130	20	44	49	66	86
	Mr. M.	124	20	40	58	—	—
	Mean:	122	13	25	41	51	65
B Acid plus dye	Mr. B.	122	3 (0)	27 (+ 2)	26 (+ 8)	54 (+11)	74
	Mr. D.	131	5 (− 6)	8 (− 7)	17 (− 3)	27 (− 1)	41 (− 1)
	Miss B.	117	7 (− 3)	17 (+ 2)	29 (+ 1)	40 (+ 2)	48 (+ 3)
	Miss B.	125	16 (− 2)	30 (+ 7)	48 (+ 5)	57 (+ 7)	73
	Miss B.	123	10 (− 2)	18 (+ 2)	30 (− 2)	42 (+ 2)	55 (− 7)
	Miss B.	126	12 (+ 2)	51 (− 7)	60 (0)	91 (− 1)	74 (+ 3)
	Mr. A.	105	0 (+11)	18 (+ 2)	76 (− 3)	—	—
	Mr. A.	127	26 (− 9)	63 (− 6)	114 (+ 2)	—	—
	Mr. A.	118	7 (+ 1)	26 (0)	45 (+12)	—	—
	Mr. A.	116	+1 (− 5)	15 (0)	42 (+ 5)	—	—
	Miss P.	121	14 (− 4)	24 (+ 4)	37 (+ 4)	60 (+11)	72 (− 4)
	Mean:	121	9 (− 1)	27 (0)	47 (+ 3)	53 (+ 3)	62 (0)
C Acid plus drainage	Mr. B.	120	4	10	23	74	—
	Mr. B.	119	13	22	31	47	50
	Mr. C.	124	19	52	98	—	—
	Mr. P.	124	9	31	42	—	—
	Mean:	122	11	29	48	60	—
D Acid plus dye plus drainage	Mr. B.	113	+2 (− 7)	29 (+12)	48 (+ 8)	68 (+14)	—
	Miss B.	131	15 (− 2)	26 (+ 4)	31 (+ 2)	37 (− 3)	44 (−11)
	Mr. A.	114	8 (− 3)	36 (+ 3)	101	—	—
	Mean:	119	8 (− 4)	30 (+ 6)	60 (+ 5)	52 (+ 5)	—

these experiments is shown by the faster rate of the emptying of the stomach when the duodenum was drained. Thus it cannot be stated that duodenal regurgitation had no effect at ordinary rates of gastric emptying. It is evident, however, that regurgitation was not essential for the reduction of the acidity of an acid test meal.

Regarding the nature of the secretion a comparison of its effects on the concentration of the dye and on the acidity shows that on the average its reaction must have been nearly neutral. However, the many discrepancies can only be explained by attributing them to technical limitations or to the action of both alkaline and acid secretions. Inspection of the average results reported by Apperly and Norris (3) shows that they too found an excellent agreement between dilution and the reduction of acidity. Even in the groups in which they were impressed by a difference their results show that the discrepancy was due to a factor operating only in the first 15 min. of the experiments. It is possible to conclude, therefore, that ordinarily the acidity of an acid test meal is reduced chiefly by dilution. It is not possible to state with any certainty whether this diluting fluid represents a single secretion or a combination of an alkaline and an acid secretion. The latter view has been upheld by Maclagan (9) and is supported by the varying discrepancies reported here.

Clearly, the present results support the contention of MacLean and Griffiths (10) that the acidity of the gastric contents is reduced by the diluting action of a neutral secretion of the stomach. However, owing to defects in available methods, such a conclusion cannot even yet be regarded as proved.

Acknowledgment

This investigation was undertaken at the suggestion of Dr. J. Wendell Macleod and appreciation is expressed for his interest and advice.

One of us, G.E.B., held a Scholarship of the Manitoba Institute for the Advancement of Medical Education and Research.

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THE REACTION OF VARIOUS TISSUES TO IMPLANTS OF A COLLAGEN DERIVATIVE[1]

By Arthur F. Battista

Abstract

This study deals with the histological reaction of subcutaneous connective tissue, muscle, peritoneum, nervous tissue, and bone to implants of a collagen derivative obtained from bovine bone, which has been called Collatissue A. The tissue response was in two stages, first, a fibroblastic proliferation about the implant and second, an infiltration by small and large mononuclear cells. Capillary proliferation occurred around the implant. The material gradually lost its normal histological structure, became amorphous, and was replaced by fibrous tissue. There was no polymorphonuclear cell response and giant cells appeared rarely. Silk, catgut, and tantalum foil were used as control materials. They induced a typical foreign body response, which included polymorphonuclear and giant cells. The antigenic properties of Collatissue A were studied to a limited extent with negative results. Collatissue A has certain physical properties, such as tensile strength and flexibility, that suggest possible surgical applications of this material.

Introduction

Surgeons have long sought for a material that would elicit very little foreign body response and that would be transformed eventually into living tissue. Such a substance might be termed a 'physiological' plastic material. The difficulty of procuring such a substance has deflected much investigation into the channel of alloplastic materials such as celluloid, various metals, paraffin, vaseline, latex, amber, and ivory. The requirements are nonabsorbability, resistance to deleterious alteration by tissue fluids, chemical inactivity, and freedom from properties that would initiate early or late tissue reaction. Each of the above-mentioned substances, as well as many others, has had its trial and supporters. The thermoplastic synthetic resins such as methyl methacrylate (Lucite and others) are the most recent materials to be studied from the point of view of surgical application.

The physical and chemical properties of collagen make it a hypothetical source of a 'physiological' plastic substance that might be utilized by the tissue elements, especially fibroblasts, and become part of the living tissue. The studies that are reported at this time are directed primarily at the histological aspects of this problem.

Historical Review

Relatively few studies of the biological properties of collagen or its denatured products (excluding gelatin) have been published. The earliest reports are descriptions of the use of decalcified chicken bone and other tubular bones as surgical drains. Protective cuffs of similar material were used about the suture line of previously sectioned nerves (12, 16, 30, 36, 1). In 1880 Senn

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[1] *Manuscript received November 16, 1948.*

Contribution from the Department of Anatomy, University of Western Ontario, London, Ont.

(33) employed antiseptic decalcified bone as an aid in the healing of aseptic bone cavities. The great difficulty was to obtain a sterile field. The material was a protein and served as an ideal medium for the growth of bacteria until it was vascularized and invaded by connective tissue (6).

Barth, in 1894 (3, 4) grafted decalcified bone to bone in the host and obtained only fibrous connective tissue union. However, Senn (33) stated that calcification did occur in some animal experiments where he had used a similar material in contact with bone.

Nageotte (26, 24, 23) isolated collagen from rat tails by a process of extraction by dilute acetic acid and precipitation with salt solution. He studied the behavior of this material in tissue cultures. He also grafted animal, human cadaver, and human living tendons into patients and found them to be tolerated well (21). Finally, Nageotte investigated the formation of collagen in tissues and isolated different fractions (27, 28). Pullinger and Pirie (31) isolated collagen from ox cornea by the Bergmann method and studied the reaction of rabbit subcutaneous tissue to the product. They concluded that collagen elicited a mild reaction that resembled chronic inflammation. They attributed this response to the insolubility of the material rather than to a chemically induced foreign body reaction.

Feriz (8, 9) studied the reaction of living tissue to a material made of collagen derived from beef tendon. He stated that the material (which was called Brocatamp) was assimilated and appeared nonirritating to the surrounding tissue when implanted in rabbits. His studies indicated that the material obtained from tendon caused less tissue reaction than did catgut or silk. Weiss and Taylor (37) employed collagen tubes in the study of nerve regeneration across gaps. No description of the preparation of these collagen tubes was given except that the tubes were "spun from fibers of dissolved and reprecipitated bovine collagen by a process developed at the Massachusetts Institute of Technology under the direction of Dr. F. O. Schmitt." Tanned and untanned tubes were prepared. They used the untanned collagen tubes in the nerve regeneration experiments but the tubes were resorbed too quickly and did not prevent loss of liquid or ingrowth of fibrous tissue. They also stated that they "have made comparative studies of the relative merits of the various collagen sleeves in ordinary nerve splicing experiments, but have been unable to reach final conclusions because of the great number of variables involved (variability of the preparations, animals, operative incidents, etc.)".

Materials and General Methods

Various authors have proposed different methods of obtaining collagen (28, 24, 9, 10). It was difficult to procure, by these methods, material that had the physical properties desired without greatly altering its chemical nature. The material used in this investigation was obtained by treating beef bone with dilute hydrochloric acid until all trace of calcium was removed. As a matter of convenience it was called Collatissue A with the thought that other collagen derivatives may form the subject of future investigations.

Bacteria and spores were destroyed in the processing, and the material was stored in 30% ethyl alcohol. It was transferred to 80% alcohol for several days before use. The material was washed thoroughly with sterile water before implantation. Since it is hydrophilic, it is likely that most of the alcohol, except that which was adsorbed, was removed. Very few infections have been encountered, and none have been related to the material. Bacteriological cultures were made of a few samples and have been negative.

Cats were used throughout the experiments since they respond well to multiple short procedures such as repeated removal of implants after varying periods of time. Intraperitoneal nembutal anesthesia was used for the implantation operation, ether for the subsequent removal procedures. Scrupulously sterile technique was the rule.

Specific Methods and Results

Series I. The Reaction of Subcutaneous Connective Tissue (and Skin) to Implants of Collatissue A

Procedure

A dorsal midline incision was made, an artery forceps passed far laterally under the skin, and a small subcutaneous pocket made by opening the forcep. Three pieces of Collatissue A, each 2 cm. × 1 cm. × 2 mm., were deposited in the subcutaneous space. The final position of the implants was on the ventrolateral aspect of the animal's body wall, far removed from the incision and its sutures. A midline abdominal incision was avoided because of the increased possibility of infection of the wound during the postoperative period.

Silk (Deknatel 6-0), catgut (type A Scanlan Plain No. 2), and tantalum foil (0.0005 in. thick) were implanted in the same manner for comparative studies.

Nine implants of Collatissue A, with surrounding tissue, were removed under sterile conditions at intervals varying between 13 and 237 days. For comparative studies there were three specimens of implanted silk (7, 20, and 28 days), three of catgut (7, 28, and 45 days), and four of tantalum foil (7, 20, 28, and 45 days). The material was fixed in Zenker–formol and stained with haematoxylin and eosin and by Mallory's method.

Observations

The reaction of subcutaneous tissue to implants of Collatissue A was relatively slight. The initial response consisted of a proliferation of connective tissue elements adjacent to the implant. This response was well developed by the 13th day. The amount of fibroblastic proliferation varied from one animal to another but in all cases a well defined capsule was formed about the implant (Figs. 1, 2). In some of the specimens implanted for long periods there was less capsular tissue present than in those of short duration. The capsule consisted of layers of fibroblasts of typical cytological structure. In later stages the fibroblasts were seen to invade the surface of the implant.

The later response of subcutaneous tissue to Collatissue A was an infiltration of adjacent tissue by small and large mononuclear cells. Very few cells of this type were present in the early stages. The time of first appearance was variable with a maximal infiltration 50 to 60 days after implantation. The mononuclear cells tended to occur in groups of 10 to several hundred. The presence of a few of these cells in the central portion of the implant attested to their amoeboid properties. Giant cells were encountered very rarely. Capillary proliferation was evident adjacent to the implant.

Collatissue A specimens that had been implanted for varying periods lost their typical histological structure in certain parts, and became amorphous. However, specimens that were in subcutaneous tissue for 237 days were readily palpable through the skin and the implant had maintained its shape and tensile strength (Fig. 2).

In contrast with the mild reaction to Collatissue, implantation of silk, catgut, and tantalum foil resulted in a typical foreign body reaction. In addition to the fibroblastic reaction, capillary proliferation, and appearance of large and small mononuclear cells, there were large numbers of polymorphonuclear cells and frequent giant cells (Figs. 4 and 5).

Series II. The Reaction of Muscle to Implants of Collatissue A

Procedure

Pieces of Collatissue A were embedded in the substance of the longus colli and longissimus dorsi muscles. The precautions followed in the case of subcutaneous implants were carefully observed. Silk, catgut, and tantalum foil were used for comparative studies. Specimens of Collatissue were removed at intervals varying between 3 and 149 days. The reaction of muscle tissue to silk, catgut, and tantalum foil after 7, 18, 28, and 45 days was studied.

Observations

The sequence of events following implantation of Collatissue A in muscle was very similar to the reaction in subcutaneous tissue (Fig. 3). The implant became encapsulated but the capsule was thinner than that produced in subcutaneous tissue (Fig. 1). The fibroblasts were more variable in orientation than in the capsule produced in the subcutaneous location. The fibroblasts invaded the substance of the implant and in the older specimens the surface areas of the Collatissue became amorphous and broken up. In one specimen of 35 days' duration, portions of the implant had disappeared, being replaced by fibrous tissue of lesser volume than the original Collatissue. In other specimens, which had been in muscle for longer periods of time, the implant remained relatively intact, demonstrating the great variability in the rate of disappearance of the implant in muscle tissue. Several factors are likely to influence the fate of the implant. The vascularity of the tissue and age of the animal are possible controlling factors. The implant appears to be altered more quickly in the young growing animal.

Large and small mononuclear cells were relatively numerous adjacent to the implant. Very few polymorphonuclear cells were seen and giant cells were encountered only occasionally. Catgut, silk, and tantalum foil, however, called forth a typical foreign body reaction. The polymorphonuclear response to silk and catgut was profuse, but somewhat less in the case of tantalum foil. Many giant cells were present about the catgut implant, some multinucleated to such an extent that they appeared as a syncytium. Occasional eosinophiles were encountered. Catgut, silk, and tantalum all incited a fibroblastic response and capillary proliferation. In general, the tissue reaction to tantalum was less marked than to silk or catgut but still much greater than to Collatissue.

B	*Bone.*	*E*	*Area in Fig. 6 enlarged and shown in Fig. 7.*
Ca	*Capsule.*	*F*	*Fibroblastic tissue.*
Ce	*Cellular elements.*	*G*	*Catgut.*
Co	*Collatissue A implant.*	*M*	*Muscle.*
D	*Dermis.*	*P*	*Panniculus carnosus muscle.*
Dc	*Cranial dura mater surface.*	*T*	*Position of tantalum foil implant.*

FIG. 1. *Series I. The reaction of subcutaneous tissue to implants of Collatissue A. Specimen No. 2 (17 days) haematoxylin-eosin (H.-E.) stain. A well defined capsule (Ca) is seen adjacent to the surface of the implant towards the dermis (D). It is definitely thicker than the capsule in earlier sections.* × *200.*

FIG. 2. *Series I. The reaction of subcutaneous tissue to implants of Collatissue A. Specimen No. 9 (237 days) H.-E. stain. The position of the implant (Co) is visible deep to the dermis (D). Capsules (Ca) are present about the surfaces of the implant. Relatively little change in the structure of the implant is observed. The edges in contact with the capsules are not broken up to any great degree.* × *30.*

FIG. 3. *Series II. The reaction of muscle to implants of Collatissue A. Specimen No. 9 (129 days) H.-E. stain. The position of the implant (Co) is seen between the muscle tissue (M). Capsule formation (Ca) is visible on each surface of the implant.* × *30.*

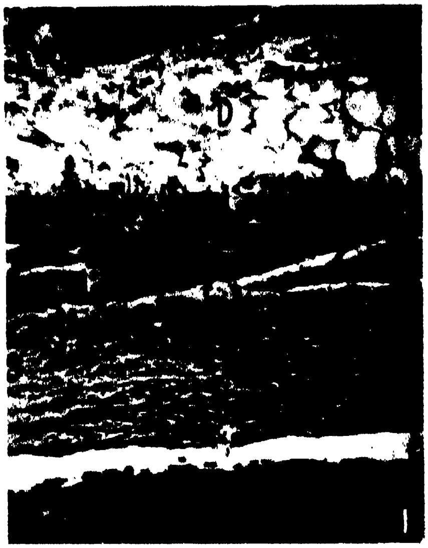
FIG. 4. *Series I. (Subcutaneous tissue) control catgut. Specimen No. 2 (28 days) H.-E. stain. The position of catgut (G) is seen. Cellular infiltration (Ce) about the implant is visible.* × *30.*

FIG. 5. *Series I. (Subcutaneous tissue) control tantalum foil. Specimen No. 1 (seven days) H.-E. stain. The tantalum foil implant (T) elicits a foreign body tissue response. A definite capsule formation (Ca) is observed. Many cellular elements (Ce) are present, especially polymorphonuclear cells.* × *125 approximately.*

FIG. 6. *Series VII. The reaction of bone and cranial dura mater to Collatissue A. Specimen No. 5 (230 days) H.-E. stain. The position of Collatissue A (Co) in relation to bone (B) is seen.* × *12.*

FIG. 7. *Series VII. The reaction of bone and cranial dura mater to Collatissue A. Specimen No. 5 (230 days) H.-E. stain. This photomicrograph is area (E), Fig. 6, seen under higher magnification. The junction of bone and Collatissue A implant is shown. Fibroblastic elements (F) form this junction. Large and small mononuclear cells constitute the limited cellular infiltration.* × *90.*

PLATE I



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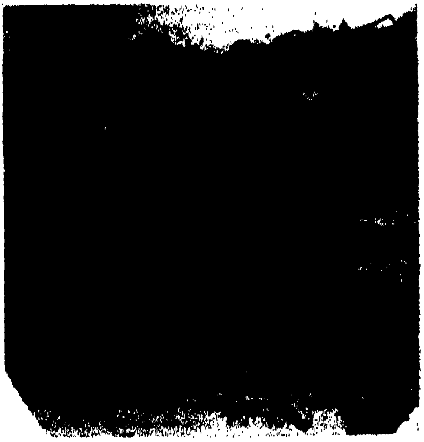

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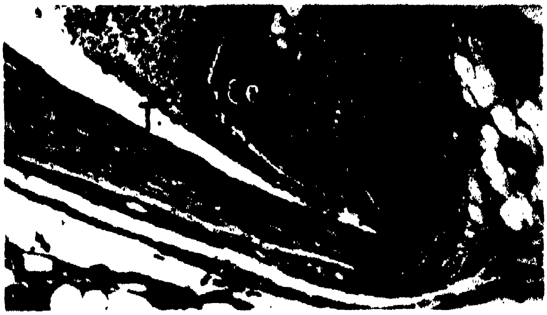

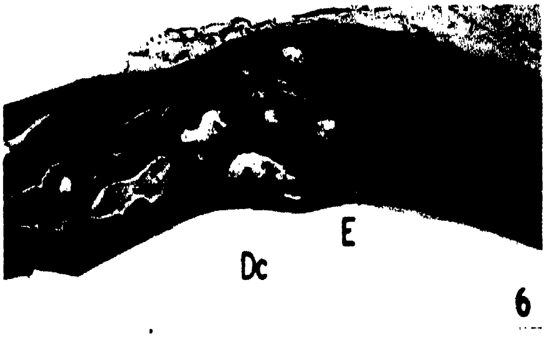

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Series III. The Reaction of Peritoneum to Implants of Collatissue A

Procedure

The ventral parietal peritoneum was exposed and an area measuring 2 cm. square removed. The peritoneal defect was filled in by a thin sheet of Collatissue, 2 mm. in thickness. It was held in place by tucking the edges between the bordering peritoneum and overlying muscle thus avoiding sutures. The fascia and skin were closed with silk. Six specimens varying in duration between 7 and 158 days were obtained.

For comparison, in a single animal a large defect was created in the parietal peritoneum and the viscera left in contact with the abdominal muscles.

Observations

There was very little tissue reaction to the peritoneal replacement. A thin fibrous capsule developed on its inner surface, consisting of typical fibroblasts arranged in a linear manner. In some specimens the omentum was adherent to the implant but it stripped away easily. In no case was there adherence of the intestine. A mild cellular infiltration of large and small mononuclear cells occurred with an occasional polymorphonuclear and giant cell. In the control animal, the intestine and omentum were bound firmly by adhesions to the muscle of the abdominal wall.

Series IV. The Reaction of Smooth Muscle of the Intestinal Wall to Collatissue A

Procedure

A small sheet of Collatissue 1 cm. × ½ cm. × 2 mm. was inserted between the muscle layers of the small intestine. The serosa was sutured with fine 6-0 silk. Two specimens, of 17 and 128 days duration were obtained.

Observations

The characteristic response to Collatissue A was observed. A fibroblastic capsule surrounded the implant, and the cells invaded the material. The surrounding tissue was infiltrated with large and small mononuclear cells, an occasional polymorphonuclear cell, and a rare giant cell. Capillary proliferation occurred in the capsule. The connective tissue of the submucosa adjacent to the implant appeared to have increased in thickness. The mucosa remained normal. There was no evidence of intestinal disturbance in the animal during life.

Series V. The Reaction of the Omentum to Collatissue A

Procedure

A piece of Collatissue 1 cm. × ½ cm. × 2 mm. was wrapped in omentum and held in place by a fine silk suture through one end of the implant. Two specimens were obtained at intervals of 17 and 128 days.

Observations

The omentum is considered a delicate tissue that responds to the slightest irritation or foreign body stimulation. Implants of Collatissue A elicited a reaction similar to that in other tissues. The usual fibrous capsule developed with invasion of the surfaces of the implant by fibroblasts. In the specimen of longer duration the Collatissue lost its normal histological structure near the surface and acquired an amorphous, moth-eaten appearance. In some areas the implant had undergone disintegration and disappeared. A cellular infiltration with large and small mononuclear cells occurred earlier than in other locations. Occasional polymorphonuclear and giant cells were seen.

Series VI. The Reaction of Cerebral Cortex and Cranial Dura Mater to Collatissue A

Procedure

A rectangular bone flap was reflected over the frontoparietal area of the skull to expose a portion of both cerebral hemispheres. An area of dura mater 1 cm. square was removed over one hemisphere. The exposed cortex was traumatized with the point of a scalpel and by the actual removal of cortical tissue by suction. There was much bleeding. A piece of very thin Collatissue A was placed over the traumatized cortex, filling in the dural defect. Three specimens, at intervals of 108, 113, and 168 days, were obtained.

For comparison, the dura was replaced by tantalum foil in several instances. In one animal the dural defect was not filled in, but the traumatized cortex was left in contact with the replaced bone flap in the case of one hemisphere, while the dural edges were drawn together over the traumatized cortex of the opposite hemisphere by two fine 6-0 silk sutures.

Observations

No adhesions were observed between the cortex and the Collatissue A dural replacement. In a few instances where the cortex had been extensively traumatized and partially removed by suction, a few strands of connective tissue were present. They stripped away very easily. The replacements were firmly fixed to the dura at the edge of the defect. The side of the implant in contact with bone bore a thin capsule of fibroblastic tissue. A few fibroblasts were seen on the inner surface but no capsule developed. The number of fibroblasts on the cerebral surface was greatest in the 168-day specimen. There was slight invasion of surrounding tissue by large and small mononuclear cells. Polymorphonuclear and giant cells were rarely seen.

In the case of tantalum foil implants the cortex was firmly adherent to the capsule that had developed on the cerebral surface of the implant. In some instances the adherent cortical tissue came away with the tantalum when the latter was reflected. Strong adhesions were present between bone and cortex where the dura had been extirpated and the cortex exposed to the bone flap.

Series VII. The Reaction of Bone and Cranial Dura Mater to Collatissue A

Procedure

A defect 1 cm. in diameter was made with a trephine over the frontoparietal area of the skull. The defect was filled by a disc of Collatissue A.

In other cases a rectangular bone defect was filled in with Collatissue A.

Five specimens, the periods varying between 20 and 230 days, were available for study.

Observations

In the 230th day specimen the Collatissue replacement remained intact. The fibrous tissue of the scalp formed a capsule on the outer surface of the implant to which the scalp was firmly fixed. There were no firm adhesions between the cranial dura mater and the Collatissue A, for the dura mater stripped away as freely as it does from bone. The implant was firmly adherent to the surrounding bone edge (Figs. 6 and 7). In older specimens it appeared that the osteocollagenous fibers of the bone were continuous with the fibers of the implant. The two materials could be differentiated by the marrow spaces of the bone and the normal histological characteristics of Collatissue. Very few cellular elements, mostly large and small mononuclear cells, were present at any stage. Polymorphonuclear and giant cells were seen rarely.

Preliminary Studies of Antigenic Properties of Collatissue A

Several preliminary tests were made to determine the antigenic properties of Collatissue A. A sterile suspension (1½ cc.) of the material (particles of almost colloidal size) was injected intraperitoneally into two mature guinea pigs. A third guinea pig received 1 cc. of the suspension intravenously and three rabbits received 2 cc. by the same route. After a period of 16 days the animals received 1 cc. of the same suspension intravenously. No reaction occurred. In a cat, a member of a series not reported upon at this time, the thorax on one side was packed with Collatissue A in order to collapse the lung. After a period of 12 months the animal received 1 cc. of a Collatissue A suspension intravenously with no immediate or delayed effects. No reaction occurred following repeated intravenous injections in this animal over a period of a month. These experiments suggest that Collatissue A has no, or very limited, antigenic properties. More extensive and precise studies are planned to determine the immunological properties of Collatissue A and similar collagen derivatives.

Discussion

Several mechanisms may be proposed for the mild response to Collatissue A when compared with silk, catgut, and tantalum foil. Some workers maintain that collagen derived from different mammals is similar chemically so that a denatured product of collagen would not be foreign to the tissues of different animals. Pullinger and Pirie (31) stated that the response of subcutaneous tissue in the rabbit to collagen derived from ox cornea resembled the tissue reaction seen in chronic inflammation. There was a predominance of lymphocytes, macrophages, and mature connective tissue fibers. They attributed the comparatively mild reaction to the insolubility of collagen and referred to the absence of features considered to be typical of a foreign body reaction.

The mechanism by which the disappearance of Collatissue A is accomplished, when this does occur, appears to be complex. The virtual absence of polymorphonuclear and giant cells, as compared with catgut and silk, tends to preclude a phagocytic process. The change from a typical architecture to an amorphous material suggests an enzymatic attack on the material resulting in increased solubility and assimilation of the altered collagen. The numerous mononuclear cells and young growing fibroblasts may play a part in this process. It is of interest to note that Feriz (8, 9) studied the fate of a commercial material made of collagen derived from beef tendon when placed in tissues of the rabbit. He stated that the material was partly absorbed by lytic activity of ferments, partly removed by phagocytosis, and partly organized either by direct infiltration of connective tissue cells or by the formation of granulation tissue.

The comparative observations on the fate of catgut, silk, and tantalum foil show how intimately polymorphonuclear and giant cells are associated with the foreign body reaction.

The first visible evidence of tissue reaction to Collatissue A is a fibroblastic response. The degree of this response varies considerably in the same tissue, and is not intimately related to the period during which the implant was embedded. Both the fibroblastic response and the amount of absorption of the material varies considerably in different tissues. There are probably multiple factors involved in this variation. Differences in the vascularity of various tissues is likely to be an important factor. It seems possible that the degree of participation of Collatissue A in the function of the tissue in which it is implanted may be important. The implant is readily absorbed in muscle in which it can serve no useful function. On the other hand, Collatissue A that replaced a peritoneal defect survived and developed a well defined fibrous capsule on its visceral surface, thus performing the useful function of repairing a peritoneal defect. In this connection the results obtained by replacing a portion of the Achilles tendon in a dog by a strip of Collatissue A is of considerable interest. After removal of a plaster cast at the end of three weeks the animal had normal use of the limb in which the repair had been carried out. The dog was observed for a period of eight months. It was impossible to detect any difference in the two limbs when the dog ran and jumped. Bone grafts are known to behave in a similar manner. Bone implanted in muscle tends to undergo decalcification and to be absorbed or it may become encapsulated by fibrous tissue. Bone grafted into bone becomes incorporated in the living dynamic tissue of its new environment.

The experience gained in this study of the biological properties of Collatissue A commends its consideration as a source material in the plastic repair of surgical defects and numerous other surgical procedures. A more extensive series of animal experiments is planned to inquire more specifically into its practical applications. Further studies of the biochemical, physical chemical, physical, and immunological properties of collagen derivatives are also required. The final evaluation of the practical value of Collatissue A in surgery can only be obtained from actual clinical trials.

Summary of Results

The various tissues of the cat that were studied in this investigation showed relatively little reaction to implants of Collatissue A. The response may be considered in two stages. There is, first, a proliferation of fibroblasts, derived from the surrounding connective tissue around the implant to form a capsule. Secondly, a cellular infiltration consisting of large and small mononuclear cells occurs. The latter resemble lymphocytes cytologically. Very few polymorphonuclear cells are seen and in some specimens there are none. Giant cells are rarely encountered. The cellular infiltration tends to be accompanied by a change in the histological structure of the implant. The material becomes amorphous and stains more lightly, these changes occurring mainly near the surface of the implant. Collatissue changed in this way is replaced by fibrous tissue.

The implanted specimens of silk, catgut, and tantalum foil called forth a typical foreign body response. In addition to the mononuclear cells there were large numbers of polymorphonuclear cells and numerous giant cells. In the case of silk and catgut especially, the giant cells were so large and the nuclei so numerous that they took on the appearance of a syncytium.

Preliminary experiments failed to detect any antigenic properties for Collatissue A.

Acknowledgments

I wish to express my sincerest thanks to Dr. G. E. Hall, Dr. H. A. Skinner, and the National Research Council, Canada, for making this investigation possible. My deepest gratitude to Dr. M. L. Barr who has been most helpful by his valued advice and aid. My thanks are offered to Mr. C. Jarvis for the photomicrographs, to Mr. J. E. Walker, and Mr. J. E. Matthews for the histological sections and assistance in operative procedures.

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ACETYLCHOLINE AND NEURONAL ACTIVITY

I. CHOLINESTERASE PATTERNS AND ACETYLCHOLINE IN THE CEREBROSPINAL FLUIDS OF PATIENTS WITH CRANIOCEREBRAL TRAUMA[1]

By Donald B. Tower and Donald McEachern

Abstract

Cerebrospinal fluid acetylcholine and cholinesterase determinations were made on 112 neurological and neurosurgical patients. Results on 15 patients with craniocerebral trauma and six psychiatric patients treated with electric shock convulsant therapy (E.C.T.) are reported in detail. Except for epileptics the only cerebrospinal fluid assays positive for acetylcholine occurred in the traumatic and E.C.T. groups, in which acetylcholine levels were 0.2 to 100+ μgm. %. In contrast to all other patients cerebrospinal fluid cholinesterases of traumatic and E.C.T. groups showed reversals of normal fraction patterns. These consisted of reduction of specific and increase of unspecified cholinesterase fractions, together with decrease of total cholinesterase activity. Acetylcholine levels and extent of cholinesterase fraction reversals paralleled the severity of cerebral damage, judged by clinical and electroencephalographic (E.E.G.) signs. Coma or semiconsciousness and depression of E.E.G. activity were associated with presence of acetylcholine and marked reversals of cholinesterase fractions in cerebrospinal fluids. In less severe cases and during recovery, when patients were disoriented and confused and E.E.G. activity was increased, cerebrospinal fluid acetylcholine decreased and disappeared, and cholinesterase values returned toward normal. Similar cerebrospinal fluid abnormalities were seen in patients after E.C.T. Changes in cerebrospinal fluid cholinesterase fraction patterns seem to be sensitive indications of extent of cerebral injury and progress toward recovery.

Introduction

In the course of studies on acetylcholine and cholinesterases in cerebrospinal fluids from 112 neurological and neurosurgical patients, interesting findings were obtained in the group with craniocerebral trauma. The literature on experimental studies of the physiology of craniocerebral trauma has been reviewed by Bornstein (3). He carried out animal experiments to investigate the role of acetylcholine in the post-traumatic state. He felt that there was a relationship of post-traumatic behavior, electroencephalographic abnormalities, and cerebrospinal fluid acetylcholine levels with the severity of concussion. He was able to reproduce these changes in unanesthetized animals by perfusion of the cortex with acetylcholine in concentrations of 1.0 to 10.0 μgm. %. With concentrations below 2.0 μgm. %, Bornstein (3) observed excitatory or synchronizing effects of acetylcholine on behavior and on the electroencephalograms (E.E.G.), while depressant effects appeared at higher concentrations.

We have reported in other papers the content and types of cholinesterases to be found in human cerebrospinal fluids (22) and the acetylcholine levels found in the same cerebrospinal fluid samples (8, 23). Three groups of

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[1] *Manuscript received December 2, 1948.*

Contribution from the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal, Canada.

This study was supported by a grant from the Rockefeller Foundation and was presented in part at the annual meeting of the American Society for Clinical Investigation, Atlantic City, N.J., May 3, 1948.

patients have been recognized. (*a*) Normal individuals and patients with various neurological disorders: In this group cerebrospinal fluid cholinesterase values were normal and acetylcholine was absent from the cerebrospinal fluid. (*b*) Epileptic patients: In this group cerebrospinal fluid cholinesterase values were normal and acetylcholine was present in the cerebrospinal fluids (in amounts from 0.02 to 5.0 μgm. %). (*c*) Craniocerebral trauma patients and patients treated with electric shock convulsant therapy (E.C.T.): In this group cerebrospinal fluid cholinesterase values were abnormal and acetylcholine was present in the cerebrospinal fluids in most instances. Clinical and E.E.G. observations of the traumatic group indicated that we were seeing for the first time in human patients phenomena that Bornstein (3, 4) had described in experimental animals.* No cerebrospinal fluid cholinesterase determinations were made by Bornstein (3, 4), so that abnormalities in the enzyme values reported here are the first to demonstrate their association with craniocerebral trauma.

Materials and Methods

Cerebrospinal fluid samples from 112 neurological and neurosurgical patients were examined for acetylcholine levels, cholinesterase values, and routine constituents. Of the samples, 80% were obtained during pneumoencephalography, the remainder during ventriculography (4%) or lumbar puncture (16%). Cell counts, Pandy tests, and protein determinations were carried out on all samples. Cerebrospinal fluid cholinesterase activities and fraction characterizations (using acetylcholine (ACh), mecholyl (MeCh), and benzoylcholine (BCh) as substrates) were done by our modification (22) of the Warburg method of Ammon (1*a*), Odom *et al.* (18) and Nachmansohn and Rothenberg (17). Cerebrospinal fluid samples for acetylcholine analysis were preserved by a modification (20) of the method of Babkin *et al.* (2) and later assayed by our modification of the method of Wait (24) on the isolated ventricle of *Venus mercenaria* (21). Electroencephalograms (E.E.G.) were taken by conventional four-channel ink-writing recorders through the cooperation of the Department of Electroencephalography.

Results

1. *Cerebrospinal Fluid Acetylcholine and Cholinesterases*

Of the 112 patients studied 15 were cases of craniocerebral trauma and nine, cases treated by electric shock convulsant therapy (E.C.T.). Only these 24 cases are considered at length in this paper. The clinical diagnoses of these patients are included in Tables III and IV.

A total of 59 cerebrospinal fluid samples from 53 nonepileptic patients were assayed for acetylcholine.** The results are summarized in Table I.

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* *The inclusion of patients treated by electric shock convulsant therapy (E.C.T.) with cases of craniocerebral trauma may seem surprising. We feel that the application of convulsive electric shocks to the cortex may be closely allied pathologically to a blow on the head (see Discussion).*

** *The results of acetylcholine assays on 60 cerebrospinal fluid samples from 57 epileptic patients are reported in a separate paper (8, 23).*

TABLE I

CEREBROSPINAL FLUID ACETYLCHOLINE ASSAYS IN 53 NONEPILEPTIC PATIENTS

Sample type	ACh positive		ACh negative		Total
Craniocerebral trauma patients	7	(35 %)	13	(65 %)	20
E.C.T. treated psychiatric patients	2	(33 %)	4	(67 %)	6*
Untreated psychiatric patients	0		4	(100 %)	4*
Others	2	(7 %)	28	(93 %)	30
	—	—	—	—	—
Total	11	(18 %)	49	(82 %)	60
Subdural fluids	0		2		2
	—		—		—
	11		51		62

* *One patient examined before and after E.C.T.*

It can be seen that 78% of the positive acetylcholine assays occurred in the traumatic groups of patients, while 65% of the negative acetylcholine assays were in the nontraumatic groups. In only two nontraumatic cases was acetylcholine found in the cerebrospinal fluid. One of these was a case of Arnold-Chiari malformation and the other a case with no central nervous system disease. With these two exceptions the nonepileptic cerebrospinal fluid samples positive for acetylcholine were restricted to cases of craniocerebral trauma (including E.C.T. cases).

Cerebrospinal fluid cholinesterase determinations were done on 111 samples from 102 patients. The results when correlated with the clinical diagnoses are summarized in Fig. 1. We have previously pointed out that there were no significant differences among the various diagnostic groups with the exception of the cases of craniocerebral trauma and those treated by E.C.T. (22). In most of the latter cases there was a tendency toward a lower rate of acetylcholine hydrolysis and a definite reversal of the usual fraction patterns, consisting of a relative decrease in the specific cholinesterase fraction (MeCh activity) and an increase in the unspecified cholinesterase fraction (BCh activity). In Table II the reversals of cholinesterase fraction patterns for the various diagnostic groups have been summarized. Of the reversals, 77% were found in the two traumatic groups of patients, whereas 91% of the cases showing no reversals were in the nontraumatic groups. Of the five nontraumatic cases with a tendency toward cholinesterase fraction reversals there were two patients with focal epilepsy and three patients with subarachnoid hemorrhage, hydrocephalus, and third ventricle tumor (with bilateral ventriculocisternostomies) respectively.

If the results of Tables I and II are compared, it is evident that patients with craniocerebral trauma showed striking differences from the rest of the patients in regard to cerebrospinal fluid analyses. These abnormalities recalled the experimental concussion studies of Bornstein (3, 4). Accordingly detailed clinical and E.E.G. studies were combined with cerebrospinal fluid analyses in following the course of these patients.

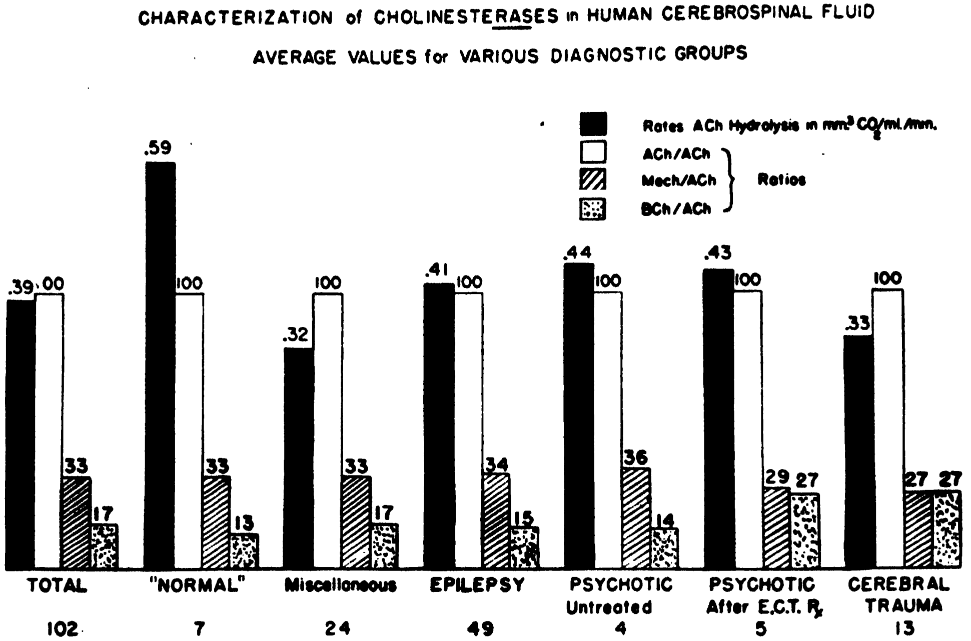

FIG. 1. *Correlation of cerebrospinal fluid cholinesterase values with clinical diagnoses in 102 patients. (Diagnoses of craniocerebral trauma and psychotic cases are given in Tables III and IV respectively. Diagnoses of miscellaneous group were: intracranial tumor, five; no CNS disease, four; cerebral thrombosis, three; headache, CNS syphilis, hydrocephalus, Arnold-Chiari malformation, adhesive arachnoiditis, and aneurysm, two each; histamine cephalalgia, trigeminal neuralgia, myasthenia gravis, Menière's disease, cerebral aplasia, idiopathic increased intracranial pressure, and multiple sclerosis, one each.).*

TABLE II

CEREBROSPINAL FLUID SAMPLES SHOWING REVERSAL OF NORMAL CHOLINESTERASE FRACTION PATTERNS

Sample type	Reversal		No reversal		Total
Craniocerebral trauma patients	12	(63 %)	7	(37 %)	19
E.C.T. treated psychiatric patients	5	(83 %)	1	(17 %)	6*
Untreated psychiatric patients	0		4	(100 %)	4*
Epileptics	2	(4 %)	47	(96 %)	49
Others	3	(9 %)	30	(91 %)	33
Total	22	(20 %)	89	(80%)	111

* *One patient examined before and after E.C.T.*

2. *Correlation of Cerebrospinal Fluid Analyses with Clinical and Electroencephalographic Observations*

To illustrate our findings four of the cases of craniocerebral trauma are briefly summarized and discussed.

(*1*) *Patient 911 JK*, ♂, *37*

Diagnosis.—Bilateral subdural hydromata, simple skull fracture of the right base and parietal bone, craniocerebral injury (Fig. 2).

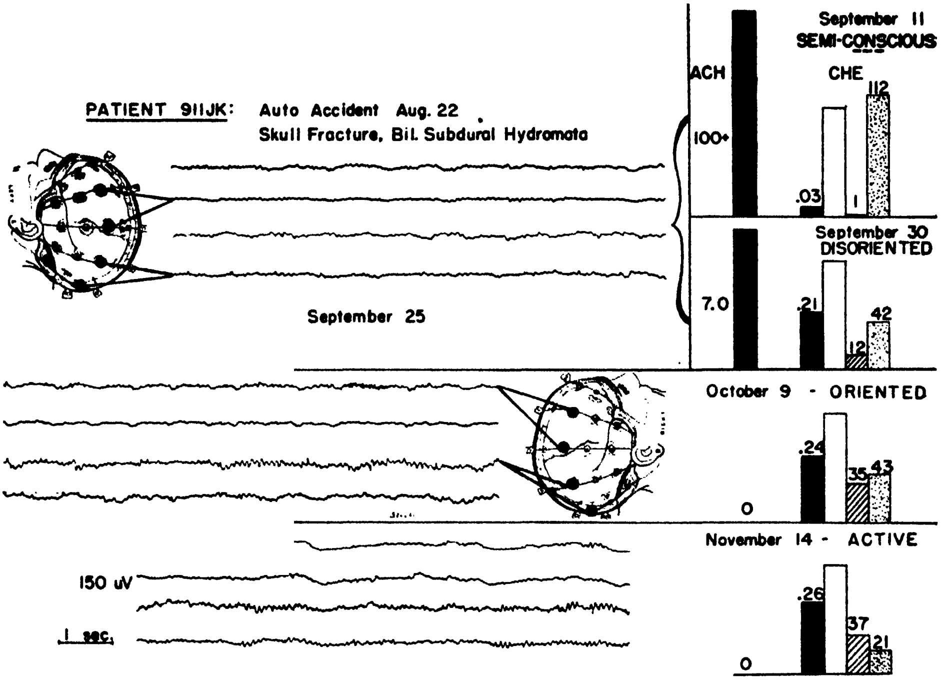

FIG. 2. *Clinical, electroencephalographic, and cerebrospinal fluid acetylcholine and cholinesterase findings on Patient 911 JK.* (*For legend see Fig. 4*).

Case abstract.—Automobile accident, Aug. 22. Rendered immediately unconscious. Admitted 24 hr. later deeply comatose with right hemiparesis, bilaterally positive Babinski signs and rectal temperature of 107° F. Comminuted fracture of right base and parietal bone demonstrated by X-ray. Bilateral subtemporal craniotomies with drainage of bilateral subdural hydromata carried out at once. Remained semicomatose for the next 20 days. Pneumoencephalogram on Sept. 11 demonstrated reaccumulation of subdural fluid. Following redrainage patient responded but was disoriented, drowsy, and at times manic and hallucinating. He became oriented on Oct. 2, and was discharged on Oct. 10. He was followed for the next year during which time he returned to active work.

The first cerebrospinal fluid sample was obtained at the time of pneumoencephalography (Sept. 11), 20 days after the accident while the patient was still semicomatose. It contained the relatively large amount of 100+ μgm. % of acetylcholine and showed a virtual absence of the specific cholinesterase fraction and a markedly reduced rate of acetylcholine hydrolysis (Figs. 2 and 3). An E.E.G. on Sept. 25 during the period of somnolence and disorientation showed extremely low voltage records from all head leads with occasional random slow waves from the right centrotemporal region. The second cerebrospinal fluid sample, taken during the same clinical period (on Sept. 30), contained 7.0 μgm. % of acetylcholine and showed some improvement in cholinesterase activity with reappearance of the specific cholinesterase fraction. One week after the patient became rational an E.E.G. (Oct. 9) showed improvement with increased amplitude, return of alpha rhythm on the right and random slow waves from the left central region. A cerebrospinal fluid sample on the same day contained no acetylcholine and showed further return of the cholinesterase activity and fraction patterns toward normal. Re-examination a month after discharge (on Nov. 14) demonstrated still further improvement in the E.E.G. with return of alpha rhythm on the left and a decrease in slow wave activity. The electroencephalographic recovery was considered in general to be very good. The final cerebrospinal fluid analysis at this time showed absence of acetylcholine and a relatively normal cholinesterase fraction pattern together with further increase in cholinesterase activity (ACh hydrolysis).

The case of *911 JK* presented the most marked changes of any we observed, but it is typical of the findings in all the traumatic cases studied. There were, of course, other factors in this case such as an initial pyrexia of 107° F. and operative reintervention. The justification for assuming a correlation between E.E.G. and clinical state and the cerebrospinal fluid acetylcholine and cholinesterase values rests with the other cases in our study.

(2) *Patient 1118 NG*, ♂, 19

Diagnosis.—Right basilar skull fracture, right epidural hematoma, cerebral contusion, craniocerebral injury (Fig. 4).

Case abstract.—Struck by hit-and-run driver on Oct. 13 and rendered momentarily unconscious. During next three hours developed headache, irritability, vomiting, and drowsiness. On examination he was found to have right basilar skull fracture by X-ray and grossly bloody cerebrospinal fluid under increased pressure. Several days later decerebrate attacks developed. Transferred to Montreal Neurological Institute on Oct. 20, where examination showed stiff neck, positive Kernig's sign, left seventh nerve paresis, paresis of left arm, and bilaterally positive Babinski signs. Ventriculogram demonstrated a fluid collection over right temporal region. Right subtemporal craniotomy and evacuation of an epidural hematoma were carried out at once. Because of his failure to respond re-explorations were carried out one and three days later, but the patient remained in a semicomatose state for the next three months. By the end of January slight improvement was evident. This became definite in the next three months. He responded to commands, walked with help, and eventually could feed himself and call for necessary articles, although he remained unable to articulate. During this period he was very restless and difficult to control. He continued to improve, regaining ability to speak, and was discharged in September.

The first cerebrospinal fluid sample was drawn about one month after admission (on Nov. 18) while the patient was still in a semicomatose state. It contained acetylcholine in a concentration of 2.0 μgm. % with a reduction of cholinesterase activity and a reversal of the normal fraction patterns (Fig. 4). E.E.G. at this time showed depression of cortical activity with bursts of high amplitude slow waves, particularly from the right temporoparietal region. Three weeks later (on Dec. 9) a second cerebrospinal fluid analysis was made. It contained 1.5 μgm.% acetylcholine and showed a

PLATE I

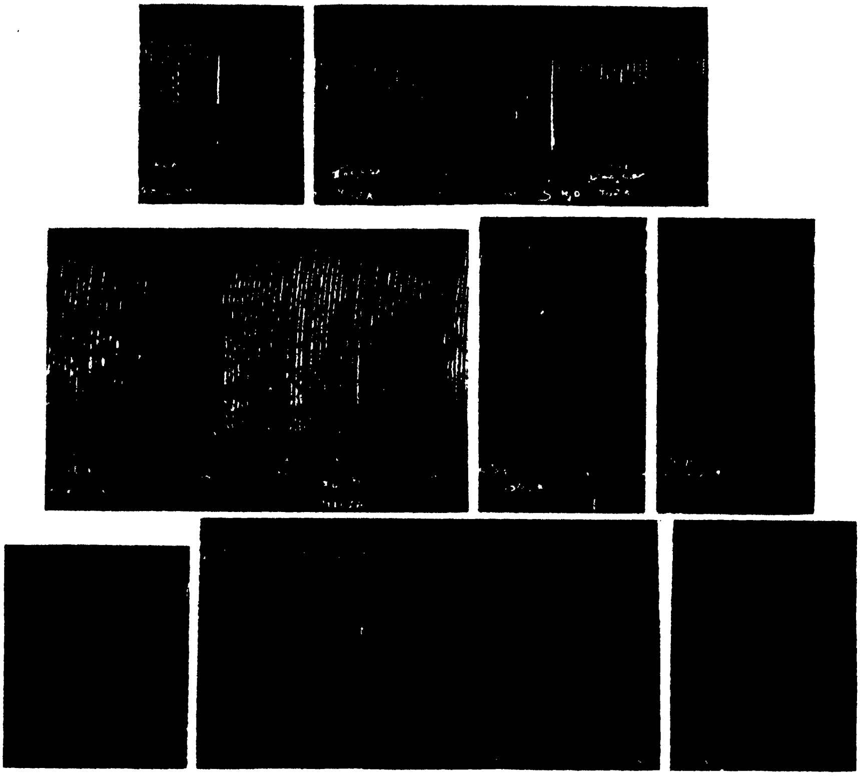

FIG. 3. *Kymograph records of cerebrospinal fluid acetylcholine assays on Patient 911 JK by the Venus heart method of assay (21).*

(Legend: ACh = acetylcholine (In concentrations of 2.5×10^{-9} *= 10.0 μgm. %*
5.0×10^{-10} *= 2.0 μgm. %*
2.5×10^{-10} *= 1.0 μgm. %*
10^{-10} *= 0.4 μgm. %)*
w = wash
911JK = CSF sample of Sept. 11 (diluted 1 : 500 and 1 : 50 as noted)
930JK = CSF sample of Sept. 30
1009JK = CSF sample of Oct. 9)

further decrease in cholinesterase activity and more marked reversal of the fraction pattern. No change in clinical state had been observed, and an E.E.G. at this time showed some deterioration from the previous record with evidence of increasing severity of right temporoparietal damage. A third cerebrospinal fluid analysis was made three months after admission (on Jan. 22) at which time clinical improvement was beginning. In the cerebrospinal fluid 1.0 μgm. % of acetylcholine was still present, but cholinesterase activity and fraction patterns showed a tendency toward improvement. The E.E.G. at this time was improved with reappearance of alpha rhythm on the left and a decrease in amplitude of the slow waves. On May 10 after three months of gradual improvement a final cerebrospinal fluid analysis showed disappearance of acetylcholine and a definite return of cholinesterase values toward normal. E.E.G. at this time demonstrated continuing improvement, characterized by reappearance of alpha rhythm on the right and a disappearance of slow waves,

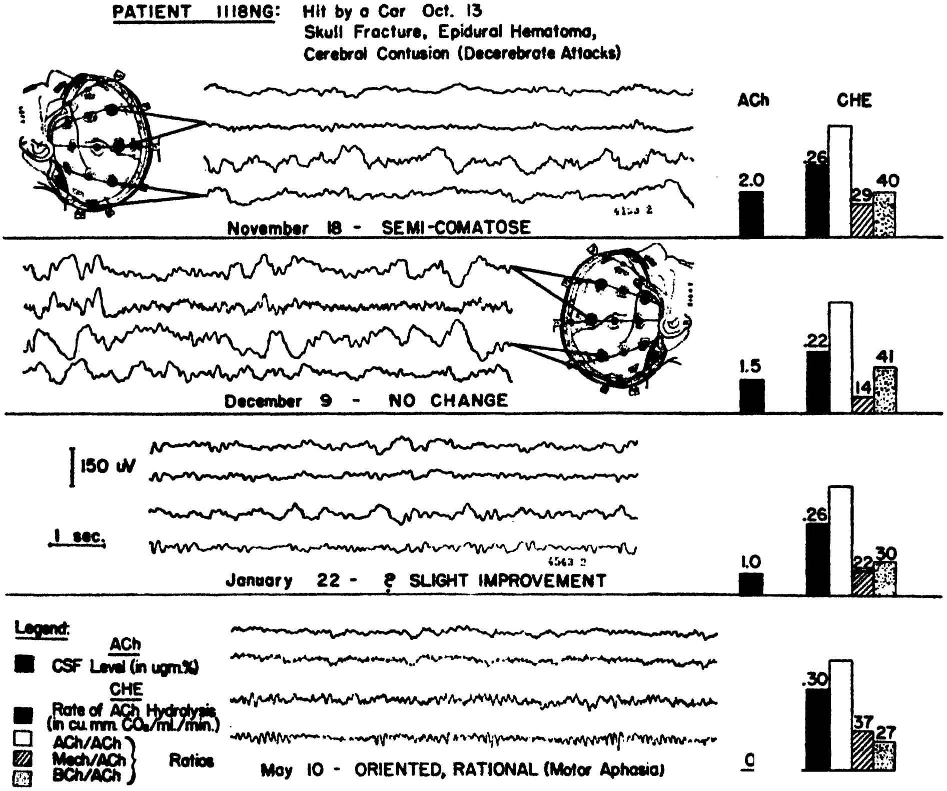

FIG. 4. *Clinical, electroencephalographic, and cerebrospinal fluid acetylcholine and cholinesterase findings on Patient 1118 NG.*

although some slow and sharp waves reversing over the right temporoparietal region suggested development of an epileptogenic focus there. The interesting feature of this case is the prolonged period of semicoma without clinical or electroencephalographic improvement during which time the abnormalities in the cerebrospinal fluid acetylcholine and cholinesterase values persisted.

(3) *Patient 0109 GB*, ♂, 29

Diagnosis.—Left temporoparietal skull fracture, bilateral subdural hematomata, craniocerebral injury (Fig. 5).

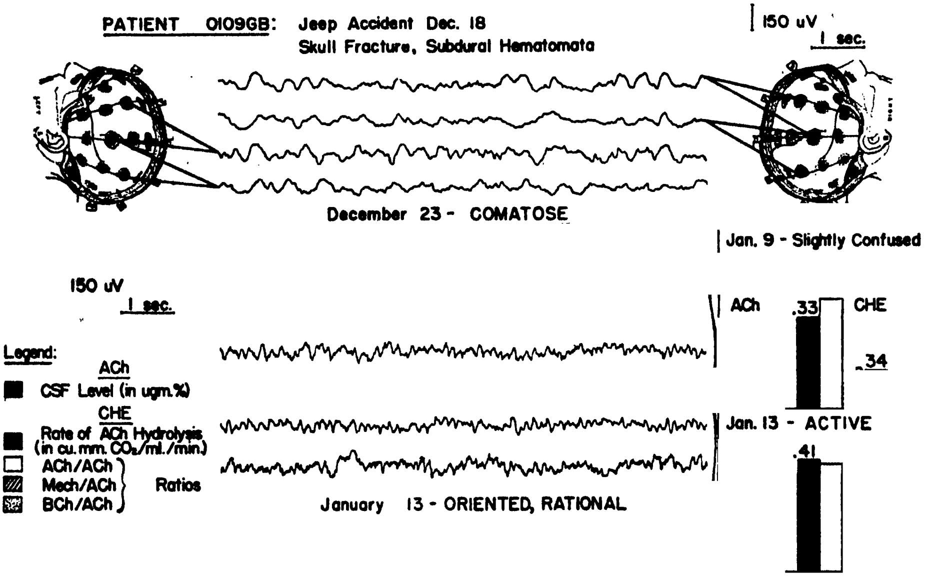

FIG. 5. *Clinical, electroencephalographic, and cerebrospinal fluid acetylcholine and cholinesterase findings on Patient 0109 GB (Note: CSF analysis dated Jan. 13 should read Jan. 20).*

Case abstract.—On Dec. 18 patient was thrown from jeep landing on his head. He remained in a restless, semicomatose state for five days at which time he was transferred to the Montreal Neurological Institute. On admission patient was comatose, with a left seventh nerve paresis, left hemiparesis, and left hyperreflexia. A linear left temporoparietal skull fracture was demonstrated by X-ray. Bilateral subdural hematomata were tapped by twist-drill holes immediately after admission, following which patient regained consciousness but remained confused and disoriented. Operative drainage of subdural spaces carried out the following day. Remained confused until Jan. 12 after which he was oriented and rational. Discharged on Jan. 23 well.

An E.E.G. at the time of admission on Dec. 23, when the patient was still comatose, showed evidence of generalized brain damage with absence of normal alpha rhythm (Fig. 5). During the period when the patient was confused and disoriented, the first cerebrospinal fluid sample was drawn (Jan. 9). It contained no acetylcholine but showed the typical abnormalities of cholinesterase values. An E.E.G. (Jan. 13), when the patient had become rational, showed marked improvement except for some bursts of four to six per second slow and sharp waves. A second cerebrospinal fluid sample (on Jan. 20) three days before discharge showed normal cholinesterase values. In this case the initial cerebrospinal fluid sampling was somewhat late in the post-traumatic course. Recovery had apparently progressed beyond the point where acetylcholine could still be detected in the cerebrospinal fluid, although the residual cholinesterase changes remained.

(4) *Patient 0216 VB*, ♂, 30

Diagnosis.—Right frontoparietal skull fracture, cerebral contusion, craniocerebral injury; multiple fractures right facial bones, contusion right eye (Fig. 6).

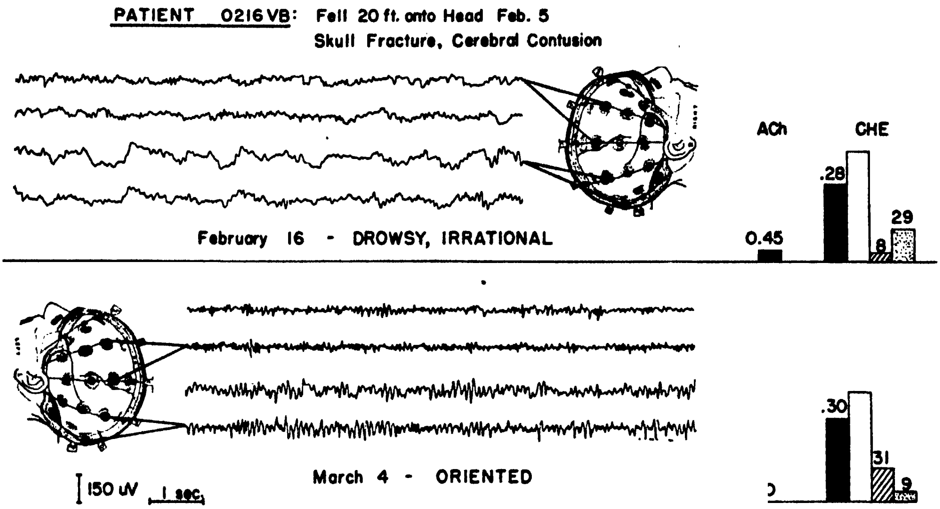

FIG. 6. *Clinical, electroencephalographic, and cerebrospinal fluid acetylcholine and cholinesterase findings on Patient 0216 VB (For legend see Fig. 5).*

Case abstract.—Four days before admission (Feb. 5) patient fell estimated 20 ft. onto head. Not rendered unconscious but subsequently became drowsy and irrational. On admission no neurological findings other than mental state of drowsiness and irrationality were elicited, but there was contusion of the right eye and evidence of multiple fractures of facial bones on the right. Right frontoparietal linear skull fracture demonstrated by X-ray. Pneumoencephalogram on Feb. 16 suggested possibility of space-occupying lesion on the right, but exploration of subdural and subarachnoid spaces was negative. Diagnosis of intracerebral hemorrhage was entertained. Gradual improvement took place, and he became clear and rational on Feb. 24. Discharged well on Mar. 5.

An E.E.G. on Feb. 16 showed very little rhythmic activity, high amplitude slow waves, and evidence of right frontoparietal damage (Fig. 6). Cerebrospinal fluid analysis at this time showed a small amount of acetylcholine and typical cholinesterase abnormalities. Ten days later (Mar. 4) at the time of discharge the E.E.G. was markedly improved with more regular alpha rhythm, decrease in slow wave activity, and higher amplitude of cortical activity. The cerebrospinal fluid sample taken at this time contained no acetylcholine and had normal cholinesterase values.

All 15 craniocerebral trauma cases studied are summarized in Table III. There is considerable variation from case to case, depending upon the time of sampling and the degree of trauma. As typified by the four cases cited (Figs. 2, 4, 5, 6) the essential features were the following: *first*, the presence initially of acetylcholine in the cerebrospinal fluid together with reduced activity and reversal of the normal fraction patterns of cerebrospinal fluid cholinesterases, so that the unspecified cholinesterase fraction predominated; *second*, the coinciding of these cerebrospinal fluid findings with depression of

TABLE III

CEREBROSPINAL FLUID ACETYLCHOLINE AND CHOLINESTERASE VALUES, ELECTROENCEPHALOGRAMS, AND CLINICAL STATES IN 15 CASES OF CRANIOCEREBRAL TRAUMA

Patient	Sex	Age	Diagnosis	Date	Cells per ml.		Pandy	Protein in mgm.%	ACh in μgm.%*	ChE rate**	ChE ratios		E.E.G. record	Clinical state
					RBC	WBC					MeCh/ACh	BCh/ACh		
909 JMcI	♂	32	Post-traumatic headache	9–9	10	2	+	35.3	0/4	0.25	43	35	Normal	Normal
911 JK	♂	37	Bilateral subdural hydromata; basal skull fracture	9–11	127	52	2+	45.1	100+	0.03	1	112	Severely depressed	Semicoma
				9–30	10	1	2+	79.5	7.0	0.21	12	42		Disoriented
				10–9	120	0	3+	97.2	0/0.3	0.24	35	43	Improved	Oriented
				11–14	2	0	+	72.5	0/1	0.26	37	21	Good recovery	Active
919 DB	♂	14 mos.	Bilateral subdural hydromata	9–19	7	0	—	19.2	0/0.4	0.25	32	15	—	Active
922 AB	♂	29	Post-traumatic headache	9–22	4	2	+	24.7	0/0.3	0.27	39	19	Rhythmic instability	Active
923 GM	♀	20	Cerebral contusion	9–23	52	0	+	29.7	—	0.26	26	20	—	Recovering
1118 NG	♂	20	Right epidural hematoma; right basilar skull fracture; cerebral contusion	11–18	34	8	—	37.1	2.0	0.26	29	40	Depressed	Semicoma
				12–9	93	21	+	57.4	1.5	0.22	14	41	Deteriorating	Semicoma
				1–22	1	0	0	—	1.0	0.26	22	30	Slight improvement	Semicoma, improving
				5–10	—	—	0	—	0/0.2	0.30	37	27	Marked recovery	Oriented
1119 JS	♂	51	Right subdural hematoma	11–19	16	2	+	64.2	0/1	0.37	26	25	Improved	Recovering
1120 AF	♂	24	Right temporal skull fracture	11–20	20	0	—	18.7	0/1	0.41	27	19	—	Recovering
0109 GB	♂	29	Left temporoparietal skull fracture; bilateral subdural hematomata	1–9	9	10	±	31.6	0/0.3	0.33	22	34	Much improved	Confused
				1–20	163	77	±	30.5	0/1	0.41	39	14		Recovering
0109 JLAP	♂	18	Cerebral contusion	1–9	14	14	+	49.1	0/0.3	0.64	34	22	Normal	Active
0110 AF	♂	58	Right temporoparietal skull fracture; right epidural hematoma; cerebral contusion	1–10	15	0	±	32.5	0/0.3	0.34	39	46	—	Semicoma
0216 VB	♂	30	Right frontoparietal skull fracture; cerebral contusion	2–16	—	—	+	44.3	0.45	0.28	8	29	Depressed	Drowsy
				3–4	15	36	0	61.0	0/0.1	0.30	31	9	Much improved	Oriented
0226 ED	♂	42	Left parieto-occipital multiple compound depressed skull fractures; left epidural hematoma; cerebral contusion	2–26	—	—	2+	97.2	0.2	0.60	20	27	Low amplitude	Manic
V1205 KJ***	♂	3 mos.	Hydrocephalus, communicating, post-traumatic	12–5	—	—	4+	527	0	2.93	4	50		
0203 CB***	♂	45	Craniocerebral injury	2–3	—	—	4+	1106	0	1.05	10	50		
911 JK***	♂	37	(See above)	9–11	—	—	4+	180	0	2.23	18	47		

* *Acetylcholine levels that are negative are expressed as 0/threshold of test object in μgm.%.*

** *Cholinesterase rate in cu. mm. CO_2/ml. CSF/min.*

*** *Subdural fluid samples.*

the E.E.G. and the clinical state; *third*, parallel improvement of E.E.G. and clinical state with disappearance of acetylcholine from the cerebrospinal fluid and return toward normal of cerebrospinal fluid cholinesterase values (the cholinesterases apparently being more sensitive indicators in this regard); and *fourth*, an apparent rough correlation of acetylcholine levels in the cerebrospinal fluid with the extent of clinical and E.E.G. abnormalities, so that with high acetylcholine levels depression was observed, while at low acetylcholine levels excitatory phenomena occurred. The observations correspond in general to the more precise experimental studies of Bornstein (3). In some cases other factors, in particular operative intervention, must be taken into account. Contamination of cerebrospinal fluids with subdural fluid, as seen in the last two cases of Table III (*V1205 KJ* and *0203 CB*), can be readily differentiated (22). In our other reports, factors of age, sex, cerebrospinal fluid cellular reaction, cerebrospinal fluid protein level, and medication of the patients have been shown to be of little or no significance for the results of cerebrospinal fluid acetylcholine and cholinesterase determinations. With the exception of cases of epilepsy and craniocerebral trauma clinical diagnoses have not had any bearing on results. The finding of acetylcholine in the cerebrospinal fluid in the presence of considerable amounts of cholinesterases has been shown to be possible because of the minimal or absent cholinesterase activity at such suboptimal substrate concentrations (8, 22, 23).

3. *Cerebrospinal Fluid Acetylcholine and Cholinesterase Studies in Nine Psychiatric Patients*

In the nine psychiatric patients available for study only cerebrospinal fluid acetylcholine and cholinesterase determinations were possible. Four of these patients received no E.C.T. treatments prior to sampling. One of these (patient *0121 EF*) was resampled after five E.C.T. treatments. The other five patients were sampled only after several E.C.T. treatments had been given.* The diagnosis and results of cerebrospinal fluid studies on these patients are summarized in Table IV.

Cerebrospinal fluid samples from the four untreated patients contained no acetylcholine and had normal cholinesterase values. In contrast the E.C.T. treated patients, with a single exception (patient *0120 HO'R*), all showed changes in cerebrospinal fluid cholinesterase fraction patterns similar to those previously described for cases of craniocerebral trauma, namely, an increase in the unspecified cholinesterase fraction (BCh activity) and a decrease in the specific cholinesterase fraction (MeCh activity). In only two of the E.C.T. treated patients was acetylcholine found in the cerebrospinal fluid samples. Patient *0121 EF* is most interesting because pre- and post-treatment samples

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* *All electric shock convulsant therapy (E.C.T.) was given at the Allan Memorial Institute of the Royal Victoria Hospital. The patients were routinely pretreated to drowsiness with intravenous sodium amytal. Electric shocks were administered from a standard Offner or Rahm machine through two electrodes, placed on vertex and temple respectively. In all these cases a convulsant dose of 700 ma. at a pulse interval of 0.7 msec. was applied for a treatment duration of two seconds.*

TABLE IV

CEREBROSPINAL FLUID ACETYLCHOLINE AND CHOLINESTERASE VALUES IN NINE PSYCHIATRIC PATIENTS

Patient	Sex	Age	Diagnosis	No. of E.C.T.	ACh in μgm.%*	ChE Rate**	ChE ratios MeCh/ACh	ChE ratios BCh/ACh
1209 TD	♂	19	Anxiety state	0	0/1	0.42	36	12
1230 EM	♂	37	Infectious psychosis	0	0/0.02	0.42	37	9
0128 AG	♀	43	Anxiety state	0	0/0.3	0.60	34	18
0121 EF	♀	48	Manic-depressive	0	0/0.1	0.32	36	16
				5	0.2	0.32	29	30
0119 RS	♀	37	Manic-depressive	6	1.0	0.59	18	32
0119 D'A	♀	37	Reactive depression	6	0/1	0.25	28	30
0120 HO'R	♀	56	Involutional melancholia	7	0/1	0.62	35	18
0120 IG	♀	32	Reactive depression	3	0/1	0.48	33	29
0130 EC	♀	36	Manic-depressive	3	0/1	0.30	36	30

* *Acetylcholine levels that are negative are expressed as 0/threshold of test object in μgm. %.*

** *Cholinesterase rate in cu.mm. CO_2/ml. CSF/min.*

were obtained. The appearance of acetylcholine in the cerebrospinal fluid and the reversal of cholinesterase fraction patterns after five E.C.T. treatments were quite striking in this patient.

The number of cases and the number of samples on each case are small but some conclusions seem warranted. The most marked cerebrospinal fluid abnormalities seemed to occur in cases with the most E.C.T. treatments. Because of the small quantities of acetylcholine found it is possible that some low acetylcholine values were missed. No explanation for the exception of patient *0120 HO'R* to the general trend of cholinesterase values in the other five patients could be found. It is interesting that this patient was the only one of the six to show no response to treatment.

Because of the findings presented in Table IV, together with the findings in accidental craniocerebral trauma (Table III) and cases of spontaneous epileptic seizures (22), we regard the cerebrospinal fluid abnormalities in E.C.T. treated cases as due to a kind of cerebral trauma produced by the electric shock. The less marked cerebrospinal fluid abnormalities in E.C.T. cases would be consistent with the milder degree of trauma delivered. In dogs given convulsant doses of electric shock Bornstein (4) found acetylcholine in the cerebrospinal fluids of several animals. Bornstein and Stern (5), Brecht and Kummer (6), and Himwich (11) have reported finding acetylcholine in the cerebrospinal fluids of patients following electric shock therapy. None have reported on the cerebrospinal fluid cholinesterases. Abnormalities in the E.E.G. (slow wave activity) (13) and atrophic gyri beneath the sites of electrode application (19) have been found after repeated E.C.T. treatments. These observations support our findings in the nine psychiatric patients studied.

Discussion

The study of patients is beset with difficulties that can often be circumvented in animal experiments. Bornstein (3) observed during perfusion of the cortex of the unanesthetized dog and cat a correlation between acetylcholine levels in the cerebrospinal fluid and the E.E.G. pattern and behavior of the animals. We have observed this in the human cases reported here. With high acetylcholine levels (over 1.0 to 2.0 μgm. %) disturbances of consciousness up to semicoma or coma together with depression of electroencephalographic activity were seen by both Bornstein (3) and us. As the level of acetylcholine decreases, patients and animals went through a period of restlessness and confusion before becoming normal. This was associated with increased activity (particularly slow and sharp waves) in the E.E.G. The cerebrospinal fluid cholinesterase changes, observed in our patients, seem to be even more sensitive indicators of the post-traumatic state than either the cerebrospinal fluid acetylcholine level or the E.E.G. The persistence of cholinesterase changes for long periods of time and their reversion to normal values coincident with clinical improvement are significant.

It is not clear what the mechanisms are for the findings reported here. Acetylcholine may be liberated because of trauma to cerebral tissue (15) or during mass neuronal discharges following trauma (25) or both. Bornstein (3) has shown that acetylcholine in the concentrations found can produce the observed effects on the E.E.G. and clinical state. In our study of epileptic patients 77% had cerebrospinal fluid samples positive for acetylcholine in concentrations lower than those seen in traumatic cases (8, 23). It has been suggested that the liberation of acetylcholine into these cerebrospinal fluids was due to the repeated trauma to the cortex of epileptic seizures. If this were so, changes in cerebrospinal fluid cholinesterases might also be expected to be present. This is not the case (22), which suggests that the appearance of acetylcholine in cerebrospinal fluids of epileptics involves a different mechanism from that seen in craniocerebral trauma.

Where the cerebrospinal fluid cholinesterase abnormalities also fit into the picture is difficult to say. We have previously suggested that the specific cholinesterase fraction is derived from nervous tissue and that the unspecified fraction is contributed by an extra-neural source (22). The increased permeability of the blood–brain barrier following trauma could account for the increase in unspecified cholinesterase. The report of Kabat *et al.* (14) is in accord with this idea. Since Cohn *et al.* (7) have shown unspecified cholinesterase to be carried in the alpha$_2$ globulin plasma protein fraction, it should be possible to apply the methods of Kabat *et al.* (14) to the further elucidation of this problem. The changes in the specific cholinesterase fraction may be due to relative inactivation of this enzyme fraction by increased destruction, decreased production, temporary inhibition, dilution with anenzymatic fluid, or any combinations thereof. It is conceivable that in certain cases craniocerebral trauma might impair the capacity of specific cholinesterase in cerebral tissue to cope with the high concentrations of acetylcholine liberated, so that the clinical course of the patient could be considerably altered (22). We feel that changes in the cerebrospinal fluid reflect changes in cerebral tissues and that attention should now be directed to an investigation of these tissues.

Further studies of the problem may result in new treatments of the post-traumatic patient. The use of atropine to counteract the effects on the E.E.G. and clinical state of abnormally high cerebrospinal fluid concentrations of acetylcholine has been suggested by Bornstein (3). Darrow *et al.* (9), Grob *et al.* (10), and Wescoe *et al.* (26) have reported studies consistent with this idea. It is also of interest that Mendel and Hawkins (16) and Hyde *et al.* (12) have used cholinesterase experimentally to alter neuronal activity. Red blood cells, rich in specific cholinesterase, are still a relatively unused by-product of plasma production, which could be made available for trial in craniocerebral trauma. We have not yet had sufficient opportunity to investigate these and other promising therapeutic approaches. The possibilities for furthering our understanding of the pathological neurophysiological processes in craniocerebral trauma and of the biochemistry of neuronal activity are many.*

Acknowledgments

We wish to express our appreciation to Drs. William V. Cone and Arthur R. Elvidge for the opportunity to study the cases of craniocerebral trauma and to Dr. Ewen Cameron, director of the Allan Memorial Institute, for the opportunity to study the psychiatric cases.

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* *Since completion of this paper the extensive review on cholinesterases by Augustinsson (1b) has appeared. It contains many results and ideas germane to the present study.*

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ACETYLCHOLINE AND NEURONAL ACTIVITY

II. ACETYLCHOLINE AND CHOLINESTERASE ACTIVITY IN THE CEREBROSPINAL FLUIDS OF PATIENTS WITH EPILEPSY[1]

By Donald B. Tower and Donald McEachern

Abstract

Cerebrospinal fluid acetylcholine assays were done on 119 samples from 109 neurological and neurosurgical patients. Of these 56 were epileptic and 53 nonepileptic patients. Acetylcholine was present in the cerebrospinal fluids of eight (15%) of nonepileptics, six of whom were cases of craniocerebral trauma (previously shown to give positive assays). In contrast 44 (77%) of epileptic patients had positive cerebrospinal fluid acetylcholine assays in amounts of 0.02 to 5.0 μgm. % acetylcholine (average 1.0 μgm. %). The presence of acetylcholine in cerebrospinal fluids of epileptics was apparently related to the frequency of seizures, the occurrence of seizures, and the extent of electroencephalographic abnormalities in these patients. Factors of cerebrospinal fluid cholinesterase values, medications, thresholds of assay methods, and types of epilepsy (with the exception of focal seizures due to intracranial tumor) seemed to be of no significance for these results. This study does not indicate whether the acetylcholine recovered in epileptic cases was directly related to the occurrence of seizures or merely a coincidental finding. The presence of acetylcholine in cerebrospinal fluids in association with epileptic seizures is considered to be significant in the light of previous studies discussed.

Introduction

There is much evidence to suggest a role for acetylcholine in the production of seizures. The present study is part of an attempt to define the biochemical mechanisms that underlie the epileptic discharge. The earlier literature has been well reviewed by Dale (18), Loewi (32), Feldberg (20), and Nachmansohn (38, 39), so that only pertinent studies will be considered here. Sjöstrand (47) was the first to observe an increase in cortical activity (by electroencephalography) with local application of small amounts of acetylcholine, eserine, and the like. Subsequent studies by Miller (36), Miller *et al.* (37), Williams and Russell (55), Brenner and Merritt (11), Merritt and Brenner (35), Chatfield and Dempsey (14), Forster *et al.* (24), Forster and McCarter (25), Bornstein (6), Hyde *et al.* (29), Grob *et al.* (27), and Freedman *et al.* (26) confirmed these findings. The effect of acetylcholine on cortical electrical activity was found to be similar to that of direct electrical stimulation or of indirect stimulation by nerve impulses and has been termed "epileptiform" in character (30).

These observations led Forster (23) to call acetylcholine unique among true convulsants in that it is normally present in the brain and can cause seizure discharges. Several specific reports may be cited in support of this statement. Bornstein (6) demonstrated the production of "epileptiform" activity in the electroencephalogram during perfusion of the unanesthetized animal cortex

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[1] *Manuscript received December 2, 1948.*

Contribution from the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal, Canada.

This study was supported by a grant from the Rockefeller Foundation and was presented in part at the annual meeting of the American Neurological Association, Atlantic City, N.J., June 15, 1948.

with 1.0 to 2.0 μgm. % of acetylcholine. Beutner (5) found that acetylcholine produced electromotive effects at phase boundaries in far smaller concentrations than any other physiological substance. Mendel and Hawkins (34) were able to inhibit nerve activity *in vivo* by injection of cholinesterases, and subsequently Hyde *et al.* (29) inhibited or delayed electrically induced convulsions by pretreatment of the animal cortex with cholinesterases. Grob *et al.* (27) and Freedman *et al.* (26) observed epileptiform activity in the electroencephalogram following administration to animals and man of the cholinesterase inhibitor, diisopropylfluorophosphate (D.F.P.). Pope *et al.* (45) found that cholinesterase activity of experimentally produced focal epileptogenic lesions was increased compared to surrounding and distant "normal" cortical tissues. On the basis of Nachmansohn's work (38, 39), Pope *et al.* interpreted their results to indicate that acetylcholine metabolism might be increased in the epileptogenic tissue. Elliott (19) could not demonstrate any abnormality of respiratory metabolism in epileptogenic cortical tissues as compared with normal cortical tissues from animals and man.

We have sought to obtain further information on the possible role of acetylcholine in the mechanism of seizures. Our method of attack was based on Bornstein's work (6) in which cerebrospinal fluids were found to contain acetylcholine following experimental concussion. The recovery of acetylcholine from the cerebrospinal fluids of animals was first reported by Feldberg and Schriever (22), and subsequently by Adam *et al.* (1), Chang *et al.* (12, 13), Chute *et al.* (15), and Berger (4). Its recovery from cerebrospinal fluids of man and animals was reported by Parrot and Lefebvre (43), Brecht and Kummer (10), Bornstein (7), Bornstein and Stern (8) following electrical stimulation of the cortex, and by Himwich (28) following metrazol convulsions.* In only three instances has the association of acetylcholine in the cerebrospinal fluid with human epilepsy been reported (41, 9, 7).

Materials and Methods

Determinations were made on 119 cerebrospinal fluid samples from 109 neurological and neurosurgical patients for acetylcholine, cholinesterases, and routine constituents. Of the samples, 80% were obtained during pneumoencephalography, the remainder during ventriculography (4 %) and lumbar puncture (16 %). Cell counts, Pandy tests, and protein determinations (16) were done on all samples. Cerebrospinal fluid cholinesterase activities and fraction characterizations were done by our modification of the Warburg method of Ammon (2), Odom *et al.* (42), and Nachmansohn and Rothenberg (40) and have been reported in a previous paper (51). Cerebrospinal fluid samples for acetylcholine levels were preserved by a modification of the method of Babkin *et al.* (3, 48) and were assayed by our modification of the method of

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* *The latter observation is of interest in view of the use of intravenous metrazol to activate epileptic foci (17, 31).*

Wait (53) on the isolated ventricle of the mollusc, *Venus mercenaria* (50). Electroencephalograms were taken on conventional four-channel ink-writing recorders in cooperation with the Department of Electroencephalography.

Results

1. *Cerebrospinal Fluid Acetylcholine and Cholinesterase Values*

Of the 109 patients studied 56 were epileptics and 53 nonepileptics.* In all cases the history of or the observation of clinical seizures were the criteria for placing patients in the epileptic group. One epileptic was not examined for cerebrospinal fluid acetylcholine. One nonepileptic has been counted twice because of a change in therapeutic status. Because of the occurrence of a single seizure during pneumoencephalography in two nonepileptic patients, it seemed advisable to classify them in both groups. The totals, therefore, in most figures and tables in this paper will read 57 epileptics and 54 nonepileptics.

Results of cerebrospinal fluid acetylcholine assays in these two groups of patients are summarized in Fig. 1. It can be seen that 44 (77%) of the epileptic patients were found to have acetylcholine in the cerebrospinal fluid

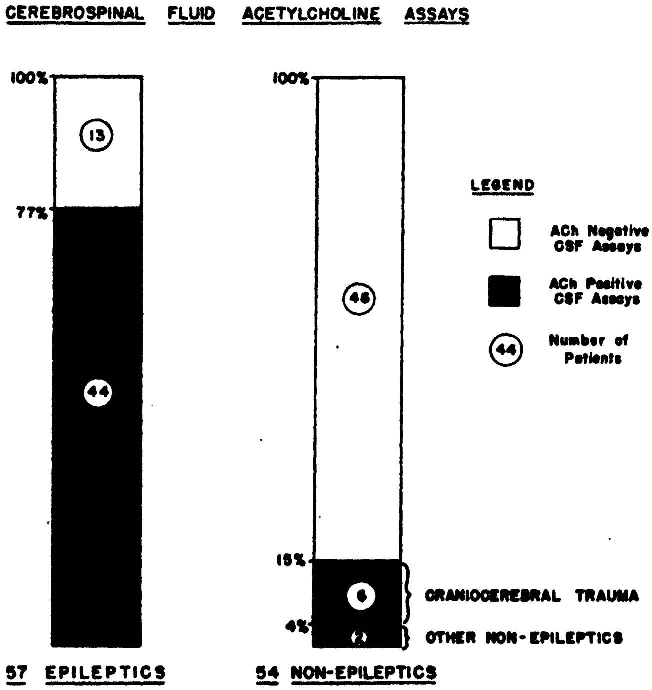

FIG. 1. *Results of acetylcholine assays on cerebrospinal fluid samples from epileptic and nonepileptic patients.*

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* *The nonepileptic group comprised the following: craniocerebral trauma, 13; psychoneurosis or psychosis, 10; intracranial tumor, five; headache (cause unknown), four; no CNS disease, four; cerebral thrombosis, three; CNS syphilis, adhesive arachnoiditis, and Arnold-Chiari malformation, two each; and Menière's disease, myasthenia gravis, trigeminal neuralgia, multiple sclerosis, cerebral aplasia, hydrocephalus, cerebral aneurysm, and undiagnosed CNS disease, one each.*

at the time of sampling. In contrast only eight (15%) of the nonepileptics gave positive assays. Of these latter eight patients, six fell into the group with diagnoses of craniocerebral trauma, in whom positive acetylcholine assays could be expected (52). Thus only two nonepileptics (4% of the total) remained as unexplained acetylcholine positives. The clinical diagnoses in these two cases were "No Central Nervous System Disease" and "Arnold-Chiari Malformation", respectively.

The amounts of acetylcholine found in the cerebrospinal fluids of the 44 positive epileptics ranged from 0.02 to 5.0 μgm. % with an average value of 1.0 μgm. %. In Table I the ranges of values have been summarized.

TABLE I

RANGE OF VALUES OF ACETYLCHOLINE FOUND IN CEREBROSPINAL FLUID SAMPLES OF EPILEPTIC PATIENTS

Range in μgm. %	No. of samples	%
0.01 - 0.25	11	24
0.26 - 0.99	17	37
1.0 - 1.99	10	22
2.0 - 5.0	8	17
Total	46	100

Average value = 1.0 μgm. %

Almost two-thirds of the cerebrospinal fluid samples contained less than 1.0 μgm. % of acetylcholine. This finding is in distinct contrast to our cases of craniocerebral trauma in whom the average was over 2.0 μgm. %. In animals subjected to experimental concussion Bornstein (6) found cerebrospinal fluid acetylcholine levels from 2.7 to 9.0 μgm. %. Brecht (9) found up to 0.1 μgm. % of acetylcholine in cerebrospinal fluids of his few epileptic cases. Attempts to correlate our cerebrospinal fluid acetylcholine levels with the severity of the epileptic process proved inconclusive. Representative *Venus* heart kymograph tracings of positive and negative acetylcholine assays on cerebrospinal fluid samples from the epileptic group of patients are illustrated in Figs. 2 and 3 respectively.

Cerebrospinal fluid cholinesterase determinations were carried out on a total of 102 patients, including 49 of the 57 epileptics. These findings are discussed in detail in another paper (51). It was found that the cholinesterase values (activity) and fraction patterns were not significantly different from those of the nonepileptic group, except for the changes previously reported in cases of craniocerebral trauma (51; 52, Fig. 1). Analysis of the epileptic group failed to demonstrate any significant correlations between cholinesterase values and types of epilepsy, seizure frequency, type of sample, presence or absence of acetylcholine, or acetylcholine level (49). The apparent paradox of the finding of acetylcholine in cerebrospinal fluids in the presence of significant amounts of cholinesterases is due to the low substrate concentrations. The acetylcholine concentrations found (Table I) were about 0.001 % of the optimal concentrations normally used to measure cholinesterase activity. At such suboptimal concentrations of substrate the cerebrospinal fluid cholinesterase activity is practically nil (51, Fig. 5).

2. *Analysis of Results in Terms of Factors not Strictly Peculiar to Epilepsy*

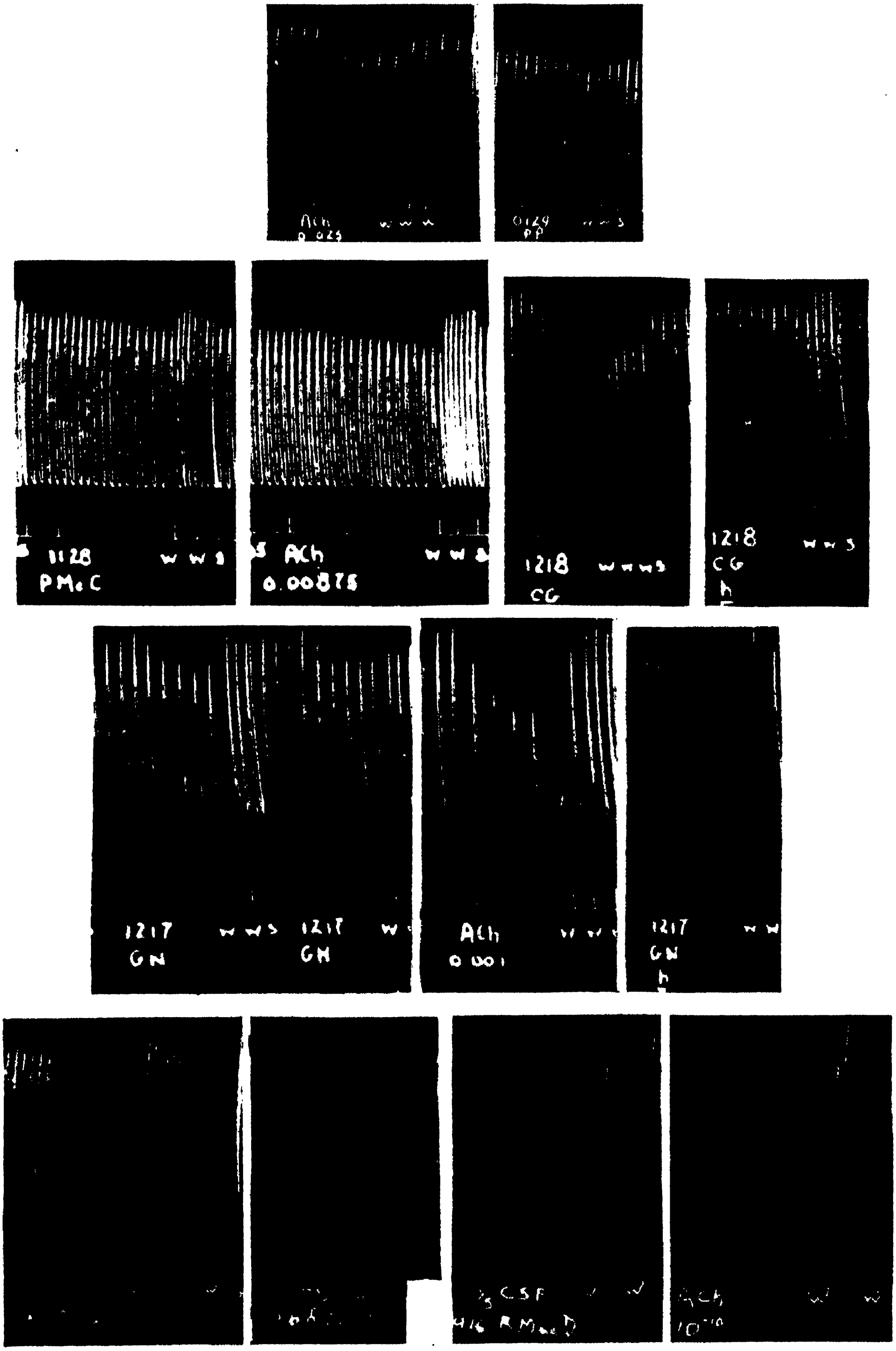
The results of cerebrospinal fluid acetylcholine assays in both the epileptic and nonepileptic groups of patients were first analyzed in terms of factors common to both groups. The factors of age and sex, of presampling medication with various sedatives, and of thresholds of the acetylcholine assay method failed to show any significant relationships to the results of acetylcholine assays on these samples. The duration of the epileptic disease process also proved to be of no significance to the results.

Analysis of the possible effect of anticonvulsant medication given to epileptic patients on cerebrospinal fluid acetylcholine assays is given in Table II. The majority of our epileptic patients are taken off medication upon admission in order to obtain satisfactory electroencephalographic records. Because of this fact the figures for medications prior to admission (*A*, Table II) are probably not significant. During admission (*B*, Table II) the percentages of acetylcholine positive and negative assays for the 18 patients receiving medications compare well both with the percentages for the 18 patients who were not on medication at any time and also with the percentages for the 40 patients who were not on medication subsequent to admission. Anticonvulsant medication, therefore, did not seem to have any significant effect on the results of cerebrospinal fluid assays for acetylcholine in these patients.

FIG. 2. *Kymograph records of positive acetylcholine assays by the Venus heart method (50) on cerebrospinal fluid samples from epileptic patients.*

(Legend: ACh = acetylcholine in concentrations of 0.025 = 1.0 μgm.%
0.00875 = 0.35 μgm.%
0.001 = 0.04 μgm.%
10^{-10} = 0.4 μgm.%
w = wash
h or hyd = hydrolyzed sample
0129PP = CSF sample on patient with cerebral seizures, unlocalized
1128PMacC = CSF sample on patient with focal left parieto-occipital seizures due to cysticercosis
1218CG = CSF sample on patient with focal right temporal seizures (cause undetermined)
1217GN = CSF sample on patient with focal left temporal seizures (cause undetermined)
916RMacD = CSF sample on patient with focal right temporoparietal seizures due to calcified ependymoma)

PLATE I


ACh
0.025
w w w
0129
PP
w w s
1128
PMeC
w w s
ACh
0.00875
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1218
CG
h
w w s
1217
GN
w w s
1217
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PLATE II

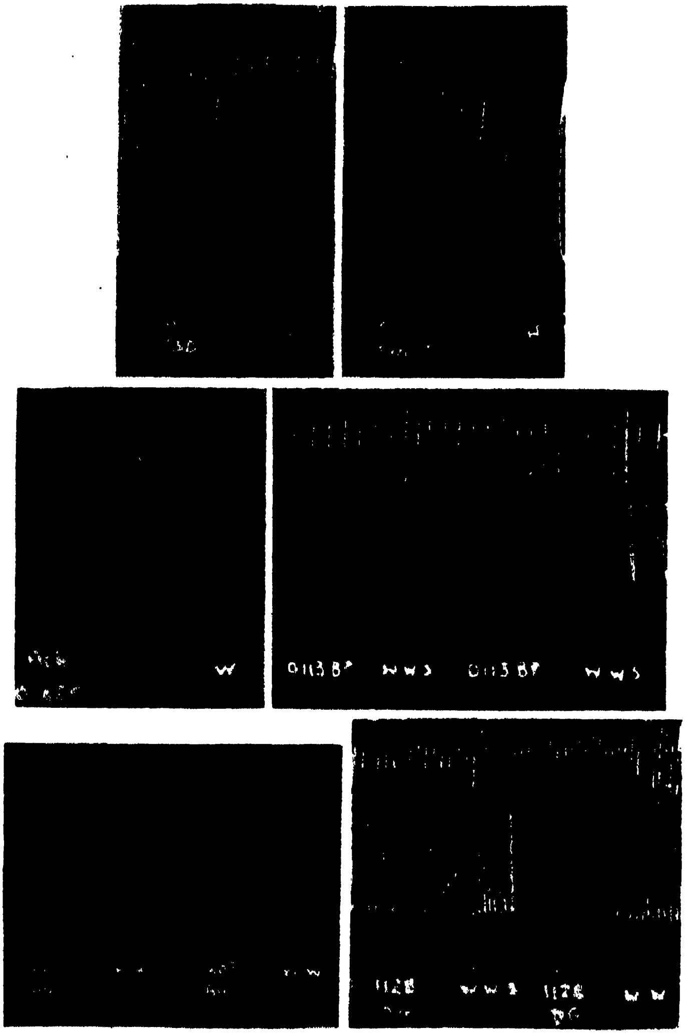

FIG. 3. *Kymograph records of negative acetylcholine assays by the Venus heart method (50) on cerebrospinal fluid samples from epileptic patients.*

(Legend: ACh = acetylcholine in concentrations of 7.5×10^{-10} *=* $3.0\ \mu gm.\%$
$0.025 = 1.0\ \mu gm.\%$
w = wash
917SD = CSF sample on patient with focal left sensorimotor seizures due to parasagittal meningeal fibroblastoma
0113BP = CSF sample on patient with cerebral seizures, unlocalized
0203AG = CSF sample on patient with focal cerebral seizures (localization in doubt) with tumor (suspected)
1128DG = CSF sample on patient with focal insular seizures (lateralization and cause undetermined))

TABLE II

EFFECT OF ANTICONVULSANT MEDICATION ON RESULTS OF CEREBROSPINAL FLUID ACETYLCHOLINE ASSAYS IN EPILEPTIC PATIENTS

Medication	Total samples	ACh positive		ACh negative	
A. *Preadmission*					
None	20	14	(70 %)	6	(30 %)
Phenobarbital	7	3	(44 %)	4	(56 %)
Dilantin	8	7	(88 %)	1	(12 %)
Phenobarbital and dilantin	11	11	(100 %)	0	
Others*	12	10	(83 %)	2	(17 %)
Total	58	45	(77 %)	13	(23 %)
B. *During admission*					
None	40	30	(75 %)	10	(25 %)
Phenobarbital	7	5	(71 %)	2	(29 %)
Dilantin	2	1	(50 %)	1	(50 %)
Phenobarbital and dilantin	6	6	(100 %)	0	
Others*	3	3	(100 %)	0	
Total	58	45	(77 %)	13	(23 %)
None (before or during admission)	18	12	(67 %)	6	(33 %)

* *Other medications were tridione, bromides, mebaral, gardenal, and sodium amytal.*

3. *Analysis of Results in Terms of Factors Peculiar to Epilepsy*

(*A*) *Types of Epilepsy*

The relationships of cerebrospinal fluid acetylcholine assays to clinical types of epilepsy given in Fig. 4 were based on Penfield's (44) classification of the epilepsies. From Fig. 4 it can be seen that the type of epilepsy was without significance except in cases of focal seizures due to intracranial tumor. In this latter group the seven negatives comprised over half the total of 13 acetylcholine negative assay cases encountered. Analyses of the types of tumors and their localizations were of no help in explaining this finding.†

(*B*) *Frequency of Seizures*

From case histories, three frequency groups for seizures prior to admission were chosen: *weekly*, one or more seizures per week; *monthly*, less than one seizure per week but one or more per month; and *yearly*, less than one

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† *Tumor types were: ependymoma, one*; hemangioma, two*; astrocytoma (unclassified), one; astrocytoma diffusum, two*; glioma (type undetermined), one; meningeal fibroblastoma, two; tumor (suspected), two*. Localizations were: frontal, three*; precentral, two; temporal, two**; insular, one*; olfactory groove, one; not recorded, two. (Each * represents one acetylcholine positive assay).*

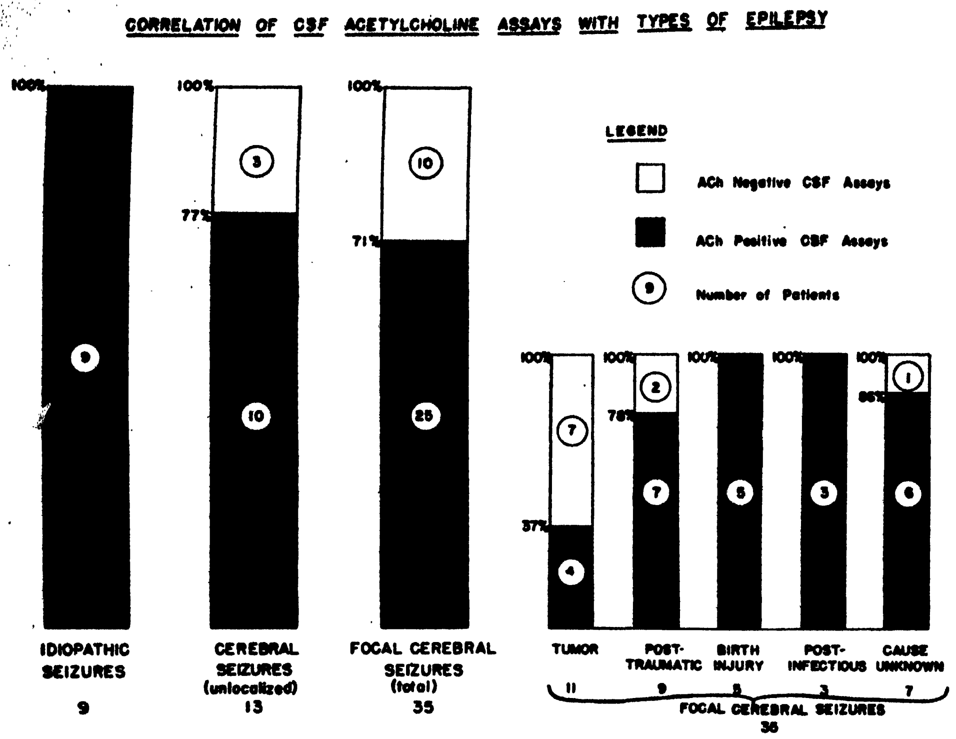

FIG. 4. *Correlation of results of cerebrospinal fluid acetylcholine assays with types of epilepsy.*

seizure per month. The relationships of cerebrospinal fluid acetylcholine assays to seizure frequency is illustrated in Fig. 5, A. In addition the frequency of patients' seizures observed during hospital admission have been correlated with acetylcholine assay results in Fig. 5, B. From these two graphs it is clear that the majority of cerebrospinal fluid samples positive for acetylcholine occurred in patients with frequent seizures, while the majority of negative assays were in patients with infrequent seizures.

(*C*) *Occurrence of Seizures and Electroencephalographic Abnormalities*

In Fig. 6 the correlation of acetylcholine assays with the time of sampling in relation to clinical seizures has been summarized. The classification of samples was arranged in ictal, postictal, and interictal groups. Only samples drawn within six hours of an observed clinical seizure were classified as postictal. All others, except true ictal samples, were designated as interictal. The graph shows that all 13 acetylcholine negative cases were restricted to the interictal group, while all 15 ictal and postictal samples were positive for acetylcholine. It is of interest to recall that of the six psychiatric cases treated by electric shock convulsant therapy (E.C.T.), which we reported in a previous paper (52), only two were positive for cerebrospinal fluid acetylcholine, although all these samples were taken within 15 min. of the electrically

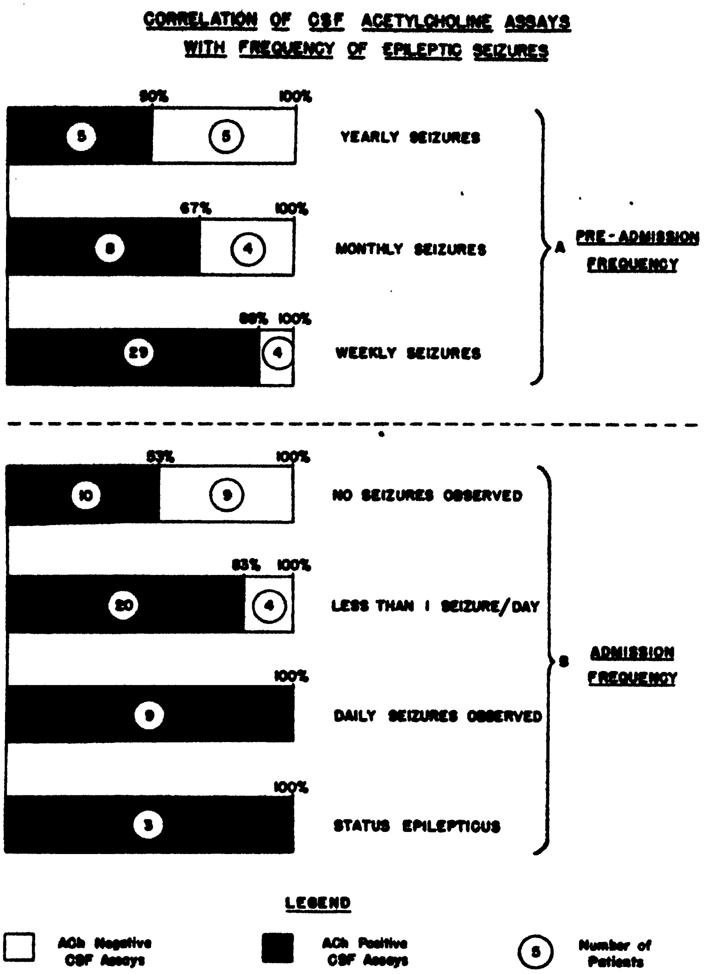

FIG. 5. *Correlation of results of cerebrospinal fluid acetylcholine·assays with frequency of clinical epileptic seizures: A—prior to admission (above dotted line) and B—during hospital admission (below dotted line).*

induced generalized seizures. This difference together with changes seen in the cerebrospinal fluid cholinesterases of the E.C.T. group of patients led us to consider them more typical of cases of craniocerebral trauma than of cases of epilepsy (52).

Because of the importance of subclinical epileptic seizures and the electroencephalographic abnormalities reflecting them, an attempt has been made to correlate the extent of electroencephalographic abnormalities with the results of cerebrospinal fluid acetylcholine assays. This is summarized in Table III. The classifications given are rather general, based only upon the predominant electroencephalographic abnormalities. The results shown in Table III are a corollary to those in Fig. 6, since the majority of acetylcholine positive assays

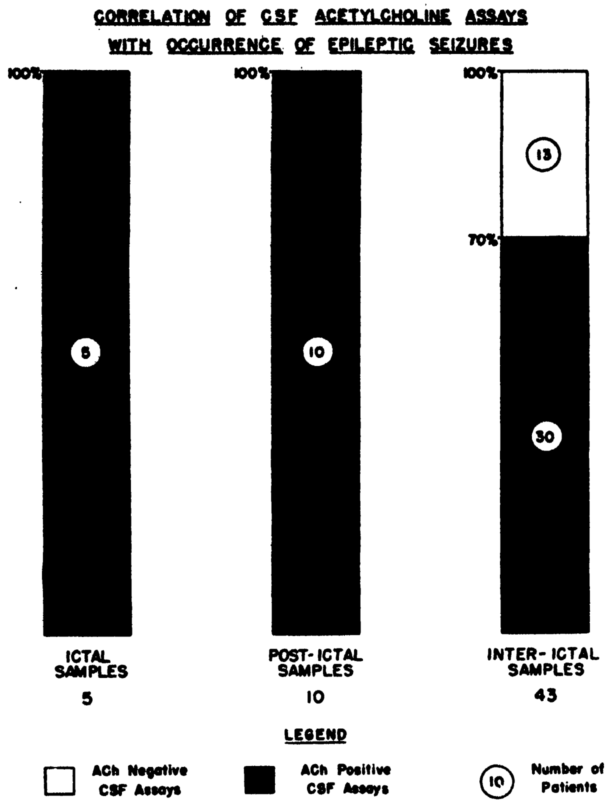

FIG. 6. *Correlation of results of cerebrospinal fluid acetylcholine assays with occurrence of clinical epileptic seizures. (Samples defined as ictal, during seizure; postictal, within six hours after seizure; interictal, all other samples.)*

TABLE III

CORRELATION OF CEREBROSPINAL FLUID ACETYLCHOLINE ASSAYS WITH PREDOMINANT ELECTROENCEPHALOGRAPHIC ABNORMALITIES

E.E.G. record abnormality	Total	ACh positive		ACh negative	
1. Paroxysmal bursts or continuous slow and sharp wave activity					
A. Idiopathic three per second wave and spike	8	8		0	
B. Other complexes	17	16		1	
	25	24	(96 %)	1	(4 %)
2. Random or diffuse slow and sharp wave activity	13	8	(62 %)	5	(38 %)
3. Borderline or no abnormality	10	5	(50 %)	5	(50 %)
4. Not tested or record unsatisfactory	9	7		2	
Total	57	44	(77 %)	13	(23 %)

were found to coincide with the more extensive electroencephalographic abnormalities, whereas the negative acetylcholine assays fell into the groups with little or no electroencephalographic abnormalities.

(*D*) *Summary*

Acetylcholine assays were made on 119 cerebrospinal fluid samples from 56 epileptic and 53 nonepileptic patients. With the exception of cases of craniocerebral trauma (52), acetylcholine was not found in the nonepileptic patients. Acetylcholine was found in the majority (77 %) of patients with epilepsy at an average level of 1.0 μgm. %, and its presence in the cerebrospinal fluid seemed to relate to the frequency and occurrence of seizures. Cerebrospinal fluid cholinesterase values, medications, assay thresholds, and other factors had no apparent bearing on these findings. It is not clear from this study whether the appearance of acetylcholine in the cerebrospinal fluids of epileptic patients is a cause or result of the seizures or merely a coincidental finding.

Discussion

There must always be an element of doubt as to the identity of a biological substance until it has been chemically isolated. The amounts of acetylcholine appearing in the cerebrospinal fluids of our cases are readily measured by the sensitive biological test employed, but they are too small to make chemical identification feasible. The assay method employed is, however, highly specific for acetylcholine (54). Of the three substances most likely to interfere, *potassium* is without effect in two to three times the concentration found in blood serum (50); *histamine* is without effect in concentrations several hundred times those actually found in cerebrospinal fluid (46); and *adenosine triphosphate* or its derivatives survive the hydrolysis to which test samples have been routinely submitted in order to prove the identity of active substance with acetylcholine (21).

Several reasons for the presence of acetylcholine in the cerebrospinal fluids of epileptic patients could be advanced. Muscular activity during a seizure might in some way be responsible for the appearance of acetylcholine in the cerebrospinal fluid. Blood cholinesterase activity could be expected to deal with such liberated acetylcholine. Furthermore our finding of positive samples from epileptic patients with *petit mal* attacks only and of a correlation between presence of acetylcholine and degree of electroencephalographic abnormality argues against the importance of muscular activity. Since the work of Lorente de Nó (33) it has been known that acetylcholine can be liberated from nervous tissue as a result of trauma. The findings of Bornstein (6) in animals subjected to experimental concussion and our own observations on human cases of craniocerebral trauma (52) confirm this fact. The question arises as to whether the results we have reported here do not represent the recovery of acetylcholine liberated as a result of the trauma to the cerebral cortex of repeated seizures. In our human cases of craniocerebral trauma we found typical changes in cerebrospinal fluid cholinesterases to be more sensitive indicators of the extent of trauma than the presence of acetylcholine in the cerebrospinal fluid (52). These cholinesterase changes were entirely lacking in the epileptic patients studied.

We conclude that acetylcholine is present in the cerebrospinal fluids of epileptic patients as a result of some other process than muscular activity or trauma, and that its presence is associated with the occurrence of the epileptic seizures. The patients in whom we were able to obtain multiple samples proved to be consistently positive. We are unable to say whether acetylcholine plays a causative role in seizures or whether its appearance in the cerebrospinal fluid is merely a reflection of other events. It may be argued that, if acetylcholine is concerned with the onset of seizures, atropine should act as a preventive. Physicians formerly used to include belladonna in the therapeutic regime for epilepsy with some good results. However, the nullifying effect of atropine on acetylcholine when applied to the cortex may be of a low order. Atropine, for example, has no effect on cholinergic transmission at the myoneural junction of skeletal muscle, whereas it can completely block transmission to visceral muscle.

Recent studies indicate that the potent anticholinesterase, diisopropylfluorophosphate (D.F.P.) increases electrical potentials of the cortex and may lead to electroencephalographic seizure patterns (27, 26). Furthermore the work on specific cholinesterase in the brain suggests that acetylcholine may be intimately concerned with epileptiform cortical discharges (34, 29, 45). In this paper we have demonstrated the presence of acetylcholine in the cerebrospinal fluids of epileptic patients in association with the occurrence of seizures. A study of epileptogenic brain tissue itself in the light of these observations would now appear to be in order.

Acknowledgment

We wish to express our appreciation to Dr. Wilder Penfield for the opportunity to study the cases of epilepsy reported in this paper.

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THE CONTENT AND CHARACTERIZATION OF CHOLINESTERASES IN HUMAN CEREBROSPINAL FLUIDS[1]

BY DONALD B. TOWER AND DONALD MCEACHERN

Abstract

Determinations of cerebrospinal fluid cholinesterase activities and fractions were made on 114 samples from 104 neurological and neurosurgical patients. Details of methods are given. Average rate of acetylcholine hydrolysis was 0.39 cu. mm. carbon dioxide per ml. CSF per min. or approximately 0.5% of the cholinesterase activity of an equal volume of blood or serum. Characterization of cholinesterase fractions, using mecholyl and benzoylcholine as substrates, showed human cerebrospinal fluid to contain specific cholinesterase with a small component of unspecified cholinesterase. Differences of blood and subdural fluid fraction patterns are demonstrated.

No correlation of cholinesterase values with cerebrospinal fluid cell counts were found. Attempts to correlate cholinesterase values with cerebrospinal fluid Pandy tests and protein levels were inconclusive, although samples with positive Pandy tests and abnormally high protein levels showed a tendency toward decrease in specific and increase in unspecified cholinesterases. No correlation of cerebrospinal fluid cholinesterase values with clinical diagnoses was demonstrated with the exception of cases of craniocerebral trauma and cases treated by electric shock convulsant therapy, where a decrease in specific and a marked increase in unspecified cholinesterase fractions were observed. At acetylcholine substrate concentrations in the "physiological range" (0.01 to 10.0 μgm. %) cerebrospinal fluid cholinesterase activities were markedly reduced or absent.

Introduction

The present study was undertaken in an attempt to provide a clearer understanding of the role of cerebrospinal fluid cholinesterases in normal and disease processes.

Several reports on cerebrospinal fluid cholinesterases have appeared in the literature. In 1930 Plattner and Hinter (37) found cerebrospinal fluid cholinesterase activity to be 1/20 to 1/25 that of blood serum cholinesterase. Vahlquist (49) and Stedman and Stedman (42) were unable to demonstrate any cholinesterase activity in spinal fluid. Ginsberg *et al.* (21) also questioned its presence there. However, Altenburger (3) reported spinal fluid cholinesterase activity to be 1/250 that of the blood. Bender (5) found 1 to 2% of the activity present in blood serum. He noted that the spinal fluid cholinesterase activity was so low that apparently not all the acetylcholine that might be liberated into it would be destroyed. Pinotti and Tanfani (36), Birkhäuser (7), and Glasson and Mutrux (22) have also found cholinesterases present in cerebrospinal fluid with about 1/200 the activity of blood and blood serum. A characterization of the types of cholinesterases present was made by Glasson and Mutrux (22). They concluded that cerebrospinal fluid contained specific cholinesterase in a concentration about 1/200 that of red cells, together with a smaller quantity of "ali-esterase." A preliminary

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[1] *Manuscript received December 2, 1948.*

Contribution from the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal, Canada. This study was supported by a grant from the Rockefeller Foundation.

report by Reiss and Hemphill (39) confirmed these findings by a somewhat different method. The four most recent studies cited have shown that there was considerable variation in the cholinesterase activities of individual spinal fluid samples, which did not seem to be well correlated with cellular reaction or protein content of the fluids. No reports have been made on large groups of patients.

Of the several methods for determining cholinesterases the one most commonly employed is the Warburg method of Ammon (4*a*), in which substrate esters are split by the enzyme to liberate the substrate acid. In the presence of bicarbonate the acid liberates carbon dioxide gas, which can be measured manometrically. Modifications of this principle for very small quantities have been used to measure the liberated acid directly by a microtitrimetric method (23), to measure the gas evolution by the "Cartesian diver" technique (8), and to measure the residual acetylcholine after completion of the reaction (14). We have used a modification of the method of Ammon (4*a*) reported by Odom *et al.* (34).

Characterization of cholinesterases depends on the demonstration of two general types of cholinesterases in the blood by Alles and Hawes (2), which was subsequently confirmed by Richter and Croft (40), Mendel and Rudney (31) and Mendel, Mundell, and Rudney (30). By the use of mecholyl (acetyl-β-methylcholine), benzoylcholine, and other choline and noncholine esters, certain differences in patterns of response were found, which were characteristic of the different types of cholinesterases. The theoretical basis of this method is schematically summarized in Fig. 1. It depends on the following

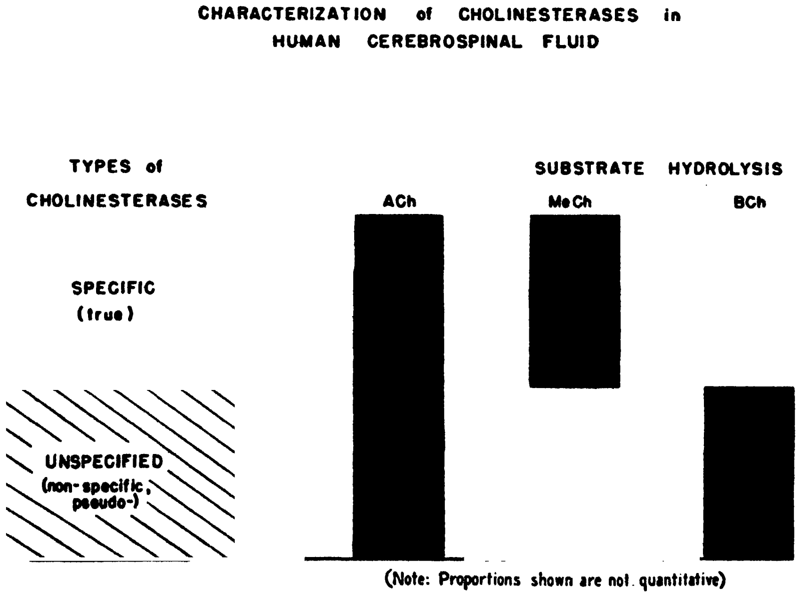
CHARACTERIZATION of CHOLINESTERASES in HUMAN CEREBROSPINAL FLUID
TYPES of CHOLINESTERASES
SPECIFIC (true)
UNSPECIFIED (non-specific, pseudo-)
SUBSTRATE HYDROLYSIS
ACh
MeCh
BCh
(Note: Proportions shown are not quantitative)

FIG. 1. *Theoretical basis of qualitative characterization of cholinesterases. (ACh acetylcholine; MeCh = mecholyl; BCh = benzoylcholine.)*

observations: (*a*) acetylcholine (ACh) is hydrolyzed by both esterase fractions; (*b*) mecholyl (MeCh) is selectively hydrolyzed by specific (or true) cholinesterase fractions; and (*c*) benzoylcholine (BCh) is selectively hydrolyzed by the unspecified (nonspecific or pseudo-) cholinesterase fractions (30, 32).* Since these are qualitative distinctions, Nachmansohn and Rothenberg (32) devised a method for making quantitative comparisons. The rates of hydrolysis of various substrates were reported as ratios of the rate of acetylcholine hydrolysis by the same sample (with ACh/ACh always equal to 100). By this method the two types of cholinesterases could be defined by their MeCh and BCh ratios as follows: *specific cholinesterase:* MeCh/ACh = 30-60; BCh/ACh = ± 1 and *unspecified cholinesterase:* MeCh/ACh = 1-5; BCh/ACh = 50-250 (32). A modification of this method has been used for characterization of cerebrospinal fluid cholinesterases in this study.

Materials and Methods

Cerebrospinal fluid samples (114 samples) from 104 neurological and neurosurgical patients were studied. Three samples proved to be subdural fluids and are considered separately from the 111 CSF samples. Of the CSF samples, 80% were obtained during pneumoencephalography, the remainder during ventriculography (4%) or lumbar puncture (16%). Care was taken to mix the total fluid volume drained from the subarachnoid spaces and ventricles before taking samples for study.

All CSF samples were examined for cell count, Pandy reaction, protein level, and acetylcholine as well as for cholinesterases. Protein determinations were done by the microturbidimetric method of Cipriani and Brophy (16). Acetylcholine assays were done by our modification of the method of Wait (50, 46) and will be reported elsewhere (47, 48, 18). The initial 30 samples were also analyzed for sugar, chlorides, and Lange curve reaction, but since it became evident that these determinations had no bearing on the main study they were discontinued.

CSF samples for cholinesterase determinations were simply refrigerated at 5° to 10° C. until used. Under such conditions the enzyme has been shown to be stable and to remain suitable for testing for several weeks (25). Our method for determining cholinesterase activity and fractions is as follows.

(1) *Apparatus*

A conventional Warburg apparatus with an electrically driven bath stirrer and shaker panel (set at 116 to 120 oscillations per min.) and a thermostatically controlled constant temperature bath (set at 37.5° C.) was used.

* *The terminology of cholinesterase fractions has become rather confusing. It has seemed to us that the most reasonable terminology is that used throughout this paper, namely, specific cholinesterase for that fraction specific to the nervous system and neuromuscular tissues and unspecified cholinesterase for that fraction found in other body tissues and fluids for which no specific function has been discovered. The terms "true," "nonspecific" or "pseudo-" suggest connotations for which no evidence has yet been offered.*

Calibrated manometers filled with Brodie's solution and fitted with calibrated conical Warburg flasks completed the apparatus.

(2) *Materials*

CSF volume of 1.5 ml. per flask was chosen on the basis of the desired rate of gas evolution for enzyme activity 0.5% that of blood serum (34). Substrate concentrations, experimentally derived on the basis of the reports of Nachmansohn and Rothenberg (32) are given in Table I. Bicarbonate solution, experimentally modified from Nachmansohn and Rothenberg (32) and Odom *et al.* (34), is also given in Table I.

TABLE I

A. *Substrate concentrations used**

Substrate	Initial concentration in %	Final molar concentration
Acetylcholine	0.5	6.9×10^{-3}
Mecholyl	1.0	1.3×10^{-2}
Benzoylcholine	1.0	1.0×10^{-2}

B. *Bicarbonate solution used**

Salt	Initial concentration in %	Parts	Final molar concentration
NaCl	0.9	80	3.1×10^{-2}
$NaHCO_3$	1.3	16	6.2×10^{-3}
$MgCl_2 . 6H_2O$	2.24	4	1.1×10^{-3}

* *For use with 0.5 ml. substrate–bicarbonate solution and 1.5 ml. enzyme solution = fluid reaction volume of 2.0 ml.*

(3) *Procedure*

CSF (1.5 ml.) was pipetted into the body of each flask and 0.5 ml. of substrate in bicarbonate solution (Table I) was placed in the side bulb. Duplicate runs were made on all samples reported here. A third flask was included in each sample run as a combined thermobarometer and autohydrolysis indicator. All flasks were saturated with 95% nitrogen – 5% carbon dioxide gas, placed in the constant temperature bath, and shaken for 20 min. to achieve equilibrium. Zero readings were taken, the substrate and enzyme solutions thoroughly mixed, and the reaction followed. Manometer readings were taken at five minute intervals for the first 20 min. and at 10 to 20 min. intervals thereafter for 60 min. reaction time. (The first 50 runs were followed for 80 min. and showed no dropping off of the plotted rate of hydrolysis.) After applying corrections for the thermobarometer–autohydrolysis deviations and the flask constants, the resulting standard gas volumes were plotted graphically against time and the rates of substrate hydrolysis derived from the slopes of the lines plotted. Cholinesterase fraction patterns were calculated from the hydrolysis rates so determined (Figs. 2, 3, 4).

1. *Human Cerebrospinal Fluid Cholinesterase Content (Activity) and Fraction Patterns*

The cholinesterase values obtained from examination of 111 cerebrospinal fluid samples from 102 patients are given in Table II. Averages of the results on these patients are illustrated in Fig. 2. It may be seen that the average rate of acetylcholine (ACh) hydrolysis was 0.39 cu. mm. carbon dioxide per

TABLE II

RANGE OF CEREBROSPINAL FLUID CHOLINESTERASE VALUES

Substrate	Rates of hydrolysis (in cu. mm. CO_2/ml./min.)	Substrate ratios (S/ACh)
Acetylcholine	0.12 – 0.89	100
Mecholyl	0.02 – 0.30	8 – 52
Benzoylcholine	0.00 – 0.19	0 – 43

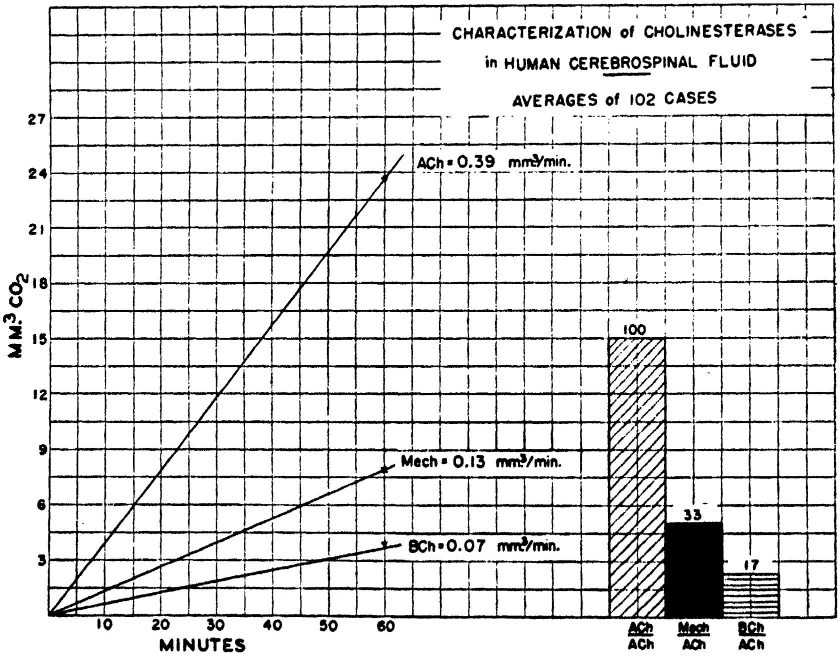

FIG. 2. *Cerebrospinal fluid cholinesterase values (For legend see Fig. 3. Rates are given per ml. of CSF).*

ml. CSF per min., and that the average substrate ratios were 33 for MeCh/ACh and 17 for BCh/ACh. By the definition of Nachmansohn and Rothenberg (32) these values characterize the cerebrospinal fluid cholinesterases as consisting mostly of a specific cholinesterase fraction with a small unspecified cholinesterase fraction. Our findings agree with the reports of Birkhäuser (7) (average ACh activity of 0.34 cu. mm. carbon dioxide per ml. CSF per min. in 46 cases) and of Glasson and Mutrux (22) (average ACh activity of 0.42 cu. mm. carbon dioxide per ml. CSF per min. in 60 cases). Reiss and Hemphill (39), using a method similar to ours in 42 mental patients, reported a comparable range of values, although their average rates of hydrolysis were somewhat higher (for ACh 0.71 to 0.79 cu. mm. carbon dioxide per ml. CSF per min.). Because of somewhat different conditions in their experiments it is not possible to compare results further.

Cholinesterase levels in normal blood samples studied by us agreed well with those reported by Odom *et al.* (34) and Nachmansohn and Rothenberg (32). One such sample is illustrated in Fig. 3. The distinction of the two principal types of cholinesterases known to exist in human blood was shown by the specific pattern of the red blood cells and the unspecified pattern of the serum. The mixture of these two fractions produced a pattern for whole blood that was analogous to that seen for cerebrospinal fluid (Fig. 2). The comparison of the rates of hydrolysis for samples of blood with the average rates for cerebrospinal fluids showed that cerebrospinal fluid cholinesterases

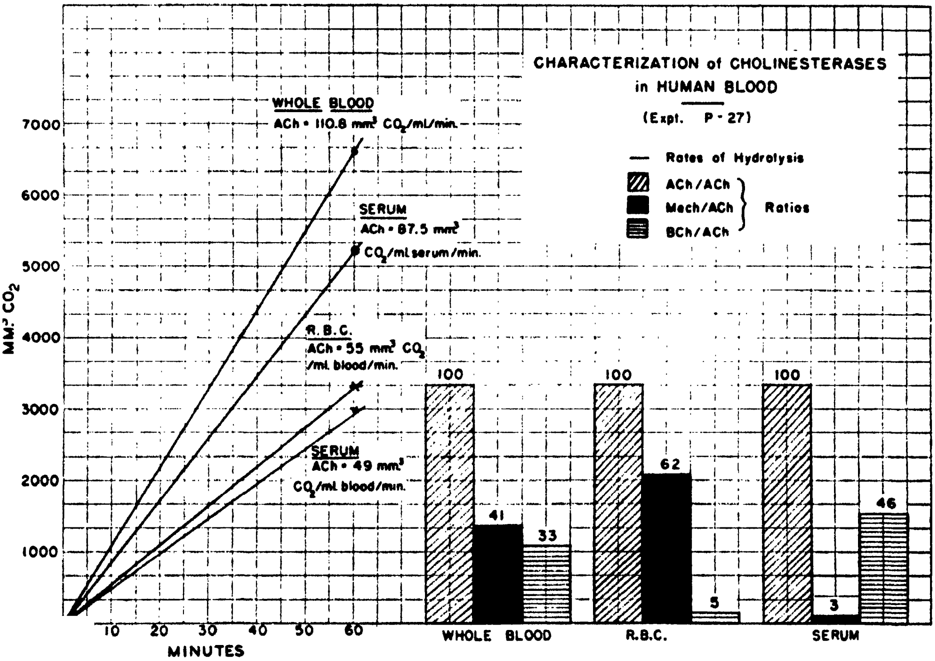

FIG. 3. *Blood cholinesterase values.*

had an average activity of about 1/225 (0.45%) that of an equal volume of serum, and 1/285 (0.35%) that of an equal volume of whole blood. No attempt was made to correlate blood and cerebrospinal fluid cholinesterase values in the same patient.

In Fig. 4 average cholinesterase values from three samples of subdural fluids obtained from subdural effusions (during ventricular puncture) contrast with the cerebrospinal fluid values obtained in other cases. The subdural fluids resembled blood serum in their cholinesterase fraction patterns, although the activity was about 2% that of the serum. Cholinesterase activity of subdural fluids (with ACh as substrate) was five times the average cerebrospinal fluid activity. These differences of subdural fluids have been of some practical value in detecting probable contamination of cerebrospinal fluid samples with subdural fluid. In our study of cases of craniocerebral trauma this has assumed considerable importance (47).

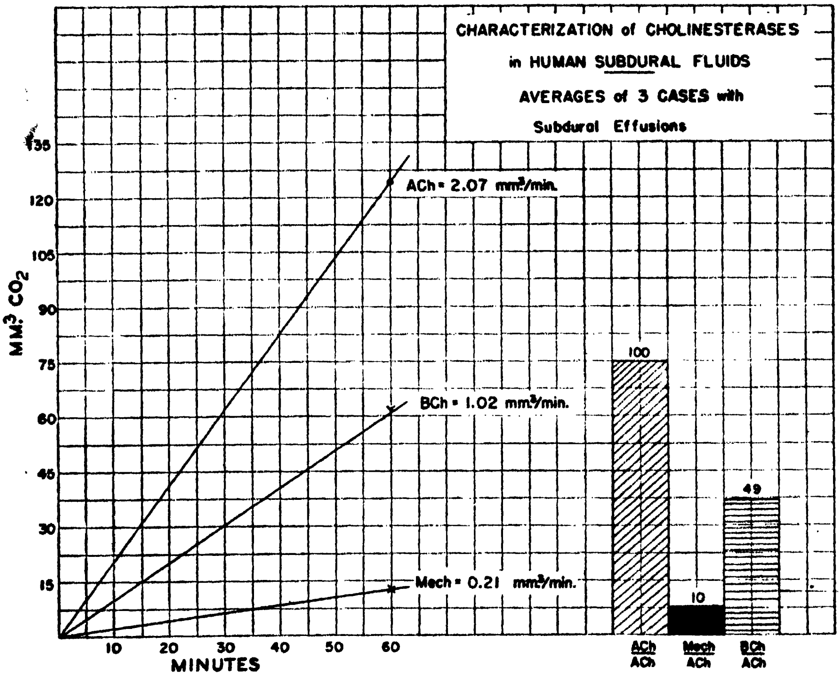

FIG. 4. *Subdural fluid cholinesterase values (For legend see Fig. 3. Rates are given per ml. of fluid).*

2. *Correlation of Cholinesterase Values with Other Constituents of Human Cerebrospinal Fluids*

Cell counts were done on 100 of the 111 cerebrospinal fluid samples. In Table III comparisons of the effects of normal and increased numbers of white cells and red cells in the cerebrospinal fluid with cholinesterase values

TABLE III

COMPARISON OF CEREBROSPINAL FLUID CELL COUNTS WITH CHOLINESTERASE VALUES

A. White blood cells

	0-6 WBC	Over 6 WBC
Number of samples	84	16
Average WBC/ml.	—	20
ChE rates (cu. mm. CO_2/ml./min.)		
ACh	0.39	0.39
MeCh	0.13	0.12
BCh	0.07	0.07
ChE ratios		
MeCh/ACh	33	31
BCh/ACh	17	17

B. Red blood cells

	0-50 RBC	50-100 RBC	100-500 RBC	Over 500 RBC
Number of samples	78	9	11	2
Average RBC/ml.	9-10	73	134	640
ChE rates (cu. mm. CO_2/ml./min.)				
ACh	0.39	0.46	0.34	0.34
MeCh	0.13	0.13	0.12	0.12
BCh	0.07	0.08	0.07	0.06
ChE ratios				
MeCh/ACh	33	28	35	35
BCh/ACh	17	17	21	18

have been summarized. No significant differences were apparent. Ginsberg *et al.* (21) were unable to find any cholinesterase activity in white blood cells. Calculation of the volume of carbon dioxide liberated by each red cell showed that a contamination of 2000 red cells per ml. would be necessary to produce a significant increase in cholinesterase activity (more than 1.0 cu. mm. carbon dioxide per hour).* None of the cerebrospinal fluid samples in our study showed a pronounced pleocytosis, and in no case did the red cell count exceed 710 per ml. Possibly, in cases of acute meningitis, differences would be found (7), but these could be due to the effects of altered permeability of the blood–brain barrier as a result of inflammation rather than to pleocytosis

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* *The rate of ACh hydrolysis by red cells (Cf. Fig. 3) was 55 cu. mm. carbon dioxide per ml. RBC per minute with a red cell count of 5.5 million per ml. This is equivalent to approximately 0.0006 cu. mm. carbon dioxide per red cell per hour.*

per se. In a similar fashion the slight decrease in the specific cholinesterase fraction seen with red cell counts of 50 and over (Table III) may have been due to serum added with the cells. Therefore, we have concluded that in the samples studied the presence of cells in the cerebrospinal fluid has not been a factor influencing cholinesterase values.

Pandy tests and ***protein determinations*** were carried out on 104 and 108 of the total of 111 cerebrospinal fluid samples, respectively. Comparisons with cholinesterase values are given in Table IV. There is a suggestion that with more strongly positive Pandy reactions there is a relative decrease in the specific cholinesterase fraction (MeCh activity) with a corresponding increase in the unspecified fraction (BCh activity). At abnormal protein levels (over 50 mgm. %) there was also a relative increase in the unspecified cholinesterase fraction and a decrease in the specific fraction. The Pandy test does not necessarily correlate with the cerebrospinal fluid protein level, but it is usually

TABLE IV A

COMPARISON OF CEREBROSPINAL FLUID PANDY REACTIONS WITH CHOLINESTERASE VALUES*

	0 Reaction	± Reaction	1+ Reaction	2+ Reaction
Number of samples	57	18	19	9
ChE rates (cu. mm. CO_2/ml./min.)				
ACh	0.38	0.43	0.38	0.41
MeCh	0.13	0.15	0.13	0.11
BCh	0.06	0.07	0.07	0.09
ChE ratios				
MeCh/ACh	34	34	34	27
BCh/ACh	16	16	19	22

* *The one sample with 3+ reaction is omitted.*

TABLE IV B

COMPARISON OF CEREBROSPINAL FLUID PROTEIN LEVELS WITH CHOLINESTERASE VALUES

	0-20 mgm. %	20-35 mgm. %	35-50 mgm. %	Over 50 mgm. %
Number of samples	26	45	20	17
Average protein level (mgm. %)	15.8	25.7	42.6	76.9
ChE rates (cu. mm. CO_2/ml./min.)				
ACh	0.32	0.39	0.43	0.43
MeCh	0.11	0.13	0.14	0.12
BCh	0.04	0.07	0.07	0.10
ChE ratios				
MeCh/ACh	34	33	33	28
BCh/ACh	13	17	16	23

positive when protein values are increased. Cohn *et al.* (17) have reported that serum (unspecified) cholinesterase is carried in Cohn's plasma protein fraction IV-6, which is 95% alpha_2 globulin and 5% albumin. Our results suggest that a significant portion of the increase in protein may be globulins, admitted because of altered permeability of the meningeal and cerebral vessels. The difficulty in attempting to correlate protein and cholinesterase values in the cerebrospinal fluids lies in the facts that both constituents represent mixtures of at least two fractions and that there are no satisfactory methods for studying the individual fractions separately. Because of the small number of abnormal samples it has not seemed justifiable to emphasize the possible significance of correlations of cholinesterase values with Pandy tests and protein levels further.

3. *Correlation of Cerebrospinal Fluid Cholinesterase Values with Clinical Diagnoses*

Average cerebrospinal fluid cholinesterase values for the various diagnostic groups are given in a previous paper (47, Fig. 1). Our primary interest has been investigating possible biochemical abnormalities in epilepsy, so that 47% of the patients fell into that group. Because of the small numbers in each of the other diagnostic groups no separate tabulations seemed justified with the exception of those shown. Rates of acetylcholine hydrolysis and cholinesterase fraction patterns for the various diagnostic groups were not significantly different with two exceptions. In cases of craniocerebral trauma the rate of acetylcholine hydrolysis was less than in other groups. And in these cases, as well as those treated by electric shock convulsant therapy (E.C.T.), there was a decrease in the specific cholinesterase fraction (MeCh activity) and a marked increase in the unspecified cholinesterase fraction (BCh activity). Of the samples, 70% in these two groups showed reversals of the usual cholinesterase fraction patterns, whereas less than 10% of the remainder showed any tendency toward such reversals. There was a definite correlation between the extent of the cholinesterase abnormalities and the degree of trauma. From these observations, together with electroencephalographic, clinical and cerebrospinal fluid acetylcholine findings, we have considered it justifiable to differentiate cases of craniocerebral trauma from the other patients studied. A detailed report of these cases will appear elsewhere (47). Detailed analysis failed to show any significant correlation of cerebrospinal fluid cholinesterase values with epilepsy or the epileptic seizure in our cases.*

4. *Cerebrospinal Fluid Cholinesterase Activity at Substrate Concentrations in the "Physiological Range"*

The finding of 0.02 to 100+ μgm. % of acetylcholine in the cerebrospinal fluids of epileptic and craniocerebral trauma patients (47, 48, 18) in the presence of cholinesterase activities capable of hydrolyzing an average of 3 μgm. of acetylcholine per ml. per min. (300 μgm. % per min.)* seemed paradoxical. Bender (5) had predicted that probably not all the acetylcholine present in the cerebrospinal fluid would be inactivated by the concentrations of cholinesterases present. Nachmansohn and Rothenberg (32) have shown that the specific cholinesterase of nervous tissue has an optimal substrate concentration (about 10^{-2} molar for ACh) and that at higher or lower substrate concentrations the cholinesterase activity drops off markedly. In our studies the final acetylcholine concentrations used in cholinesterase determinations were about 125,000 times the average of 1.0 μgm. % acetylcholine in epileptic cerebrospinal fluid samples (48, 18).

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* *Because Pope et al. (38) had reported increased cholinesterase activity in tissues from experimentally produced focal epileptogenic lesions, it seemed that some changes might be expected in cerebrospinal fluid cholinesterases as well. No differences were uncovered among the types of epilepsy, seizure frequency, types of spinal fluid samples, presence or absence of acetylcholine in samples, or the level of acetylcholine present in the cerebrospinal fluid (18, 48).*

Accordingly a series of experiments was carried out to test the cholinesterase activities of cerebrospinal fluids at optimal substrate concentrations and at concentrations down to those actually encountered in clinical experience. Fig. 5 shows the curve derived from these experiments. At substrate concentrations below 150 μgm. % the manometric method is no longer able to detect cholinesterase activity. For this range a modification of the method of Bullock *et al.* (14) was used.** From this set of experiments it seems clear

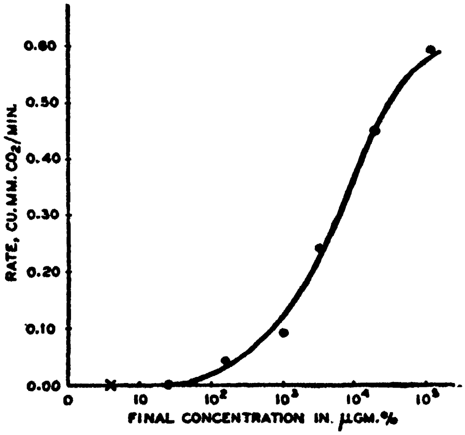

FIG. 5. *Curve of variation in cerebrospinal fluid cholinesterase activity with substrate concentrations. Rates of ACh hydrolysis plotted against final substrate concentrations.* (● = *Warburg method;* × = *Venus heart method.*)

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* *Cholinesterase activity of 1.0 cu. mm. carbon dioxide per ml. CSF per min. is equivalent to hydrolysis of 7.3 μgm. of acetylcholine per ml. CSF per min.*

** *Amounts of acetylcholine to give final concentrations of 0.2 to 4.0 μgm. % were added to cerebrospinal fluids, previously shown to be free of acetylcholine. The samples were incubated at 37.5° C. for one hour. Control samples were taken at the start of the experiments and at 30 and 60 min. intervals of incubation time. Residual acetylcholine concentrations were preserved by the method of Tower (45) until they could be assayed on the isolated ventricle of Venus mercenaria (46). No attempt was made to control all the factors involved such as pH, loss with bicarbonate and the like, but results in 12 experiments were sufficiently consistent to make us feel that the method served the purposes of this study.*

that at substrate concentrations in what may be termed the "physiological range" the cholinesterase activity of cerebrospinal fluids in most cases was zero. These findings are consistent with those of Nachmansohn and Rothenberg (32, Fig. 2). We conclude that the activities of cerebrospinal fluid cholinesterases were not significant factors in assays for cerebrospinal fluid acetylcholine, which we report elsewhere (47, 48, 18).*

Discussion

That specific cholinesterase is the principal fraction of human cerebrospinal fluid is of interest. Mendel and Rudney (31) and Nachmansohn and Rothenberg (32) have shown that the cholinesterase of nervous tissue is specific cholinesterase. It is noteworthy that the enzyme in cerebrospinal fluid, which bathes this tissue, should also be specific cholinesterase. This may throw further light on the sources of cerebrospinal fluid and its usefulness in reflecting events in the central nervous system. Since the red cell is the only extraneural source of specific cholinesterase (31, 40, 32), it seems probable that the source of specific cholinesterase in cerebrospinal fluid is the central nervous system. The enzyme is a protein, and it follows that one fraction at least of the spinal fluid protein is contributed by nervous tissue. This is not a new concept, and certainly the studies of Spiegel-Adolph (43), Spiegel *et al.* (44), Sawyer (41), Hyden (27, 28), and Hamberger and Hyden (24) support this view.

Since there is little or no unspecified cholinesterase in nervous tissue, its presence in the cerebrospinal fluid suggests an extraneural source and the same for the protein fraction in which it is carried. The finding of increased unspecified cholinesterase fractions with positive Pandy tests and abnormally high protein levels (particularly in cases of craniocerebral trauma) complement one another to indicate a probable increase in the contribution of the extraneural protein fraction or fractions, which is presumably due to alterations in permeability of the blood–brain barrier. The recent report of Kabat *et al.* (29) fits in with this concept. In addition in craniocerebral trauma the specific fraction of cerebrospinal fluid cholinesterases is reduced. These observations have led us to suggest that cerebrospinal fluid cholinesterases may be sensitive indicators of certain abnormalities of central nervous system function (47).

One other interesting problem is revived by this study, namely, the problem of cholinesterase excess in the central nervous system (20). The presence of amounts of specific cholinesterase apparently more than sufficient to hydrolyze all the acetylcholine that might be liberated into it has puzzled neurophysiologists. Free acetylcholine has been reported in the cerebrospinal fluid by a number of investigators (19, 1, 33, 15, 12, 6, 13, 35, 9, 10, 11, 26, 47, 48, 18). The explanation of this apparent paradox, as far as the spinal fluid cholinesterases are concerned, has been offered in this study. It depends on the fact that at suboptimal concentrations of substrate in the "physiological range" specific cholinesterase activity is markedly reduced or absent. Nachmansohn and Rothenberg (32) have shown that a similar situation exists in nervous tissue, but this seems to have been overlooked. The results of our experiments suggest the importance of the same approach to the study of specific cholinesterase activity of nervous tissue.

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* *Since completion of this paper the exhaustive review of cholinesterases by Augustinsson (4(b)) has been published. The experimental observations reported by him for tissue and blood cholinesterases corroborate and expand the findings and conclusions presented here for cerebrospinal fluids.*

We raise the possibility that the apparent excess of cholinesterase is not an excess at all, but that it is the amount necessary to deal with suboptimal acetylcholine concentrations usually encountered. The fact that the optimal substrate concentration for the enzyme is far above conceivable physiological ranges is intriguing. Could it be that the enzymes serve another purpose, that of dealing with massive amounts of acetylcholine liberated under certain pathological conditions? It might be logical to think of specific cholinesterase as possessing these dual functions to cover an extensive range of substrate concentrations (47). Certainly a critical review of the role of specific cholinesterase in neuronal activity is indicated.

Acknowledgment

The authors wish to express their appreciation for the helpful counsel of Dr. K. A. C. Elliott.

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SOME EFFECTS OF DIISOPROPYL FLUOROPHOSPHATE (DFP) AND FLUOROACETATE ON THE CENTRAL NERVOUS SYSTEM[1]

By Andrew Kelen and Donald McEachern

Abstract

Attempts were made to produce convulsions in cats by intracarotid and intracisternal injections of diisopropyl fluorophosphate (DFP). No convulsions were obtained in either instance. Systemic symptoms appeared, however, and plasma cholinesterase was sharply reduced. Intracarotid DFP produced prolonged ipsilateral myosis and salivation. E.E.G. changes on the side of the injection were found in one of two trials. Intracisternal DFP caused prolongation (threefold) of the pentothal anesthesia. The animals, upon awakening, showed a temporary loss of sensation in the forelimbs and cornea with weakness of the hind limbs and a waddling gait. Intracisternal fluoroacetate produced curious generalized seizures. These were classified as scissors spasms, scratching seizures, and myoclonic jerks. They appeared after about three-quarters of an hour, in contrast to the latent period of two to three hours after intravenous injection. Seizures persisted for hours unless stopped with nembutal. Cerebrospinal fluid (CSF) was tested for acetylcholine (ACh), the minimum amount detectable during our experiments being 0.4 to 3.0 μgm. %. After intravenous injections of 1.5 mgm. per kgm. of DFP preceding convulsions produced by thujone, small amounts of ACh appeared in three out of four experiments. Intravenous injection of 5.0 mgm. per kgm. of DFP resulted in 3.0 μgm. % of ACh in the CSF. No ACh was found after the other procedures described.

Introduction

The production of epileptic seizures through excess accumulation of acetylcholine (ACh) in brain tissue would be of great interest. It is natural to seek one common mechanism that might mediate convulsive seizures of many different kinds. The acetylcholine–cholinesterase system naturally comes to mind. The possibility that ACh is responsible for normal synaptic transmission in the brain is supported by considerable evidence. This has been reviewed by Feldberg (16) and Nachmansohn (29).

Cholinesterase (ChE) activity is increased in tissue from focal epileptogenic cerebral cortex in humans and monkeys. This suggests that the rate of liberation of ACh in such tissue is greater than normal (34). Recent studies have demonstrated the presence of ACh in the cerebrospinal fluid (CSF) of humans who have frequent epileptic seizures (12). ACh may therefore have a significant role in the production of convulsions. We have tried to produce a chronically epileptic animal by injecting the anticholinesterase diisopropyl fluorophosphate (DFP) into the carotid artery of cats. We hoped to restrict the action of this potent chemical to the brain, or even to one hemisphere of the brain, and thus to avoid its systemic effects. Any passage of DFP into the general circulation could be determined by the blood ChE levels before and after injection of the drug.

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[1] *Manuscript received December 2, 1948.*

Contribution from the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal, Que.

In other experiments, DFP was injected into the cisterna magna, again with the object of restricting its action to the brain.

Intracisternal injections were also carried out with sodium fluoroacetate, a drug that is known to produce seizures in some animals when given systemically (9).

Finally, experiments were made to determine the presence of ACh in the CSF of animals following injection of DFP and fluoroacetate, and after seizures produced by metrazol and thujone and by electric shock.

Methods*

1. *Carotid Artery Injections*

Using aseptic precautions and with the test animals under nembutal anesthesia (32 mgm. per kgm. of body weight), DFP** was injected into the exposed common carotid artery of six animals in amounts varying from 0.9 to 1.8 mgm. per kgm. The DFP was in an *M*/100 solution in propylene glycol. Immediately before injection, the DFP solution was diluted to 10 ml. with normal saline. A seventh cat was given a solution of propylene glycol without DFP, as a control.

2. *Cisternal Injections*

Cats were anesthetized with sodium pentothal (20 to 25 mgm. per kgm. intravenously). The back of the head and neck were shaved and CSF was withdrawn from the cisterna magna and replaced by freshly prepared solutions of DFP or sodium fluoroacetate*** ranging from minimal to lethal doses and contained in 1 ml. of normal saline or bicarbonate–Ringer solution. Control injections of saline and bicarbonate–Ringer solution were made. A second cisternal puncture was made under nembutal anesthesia (22 mgm. per kgm.) from 15 min. to five hours after the first puncture, and CSF was removed for ACh assays. Control assays were done on CSF drawn prior to intracisternal injections in 12 experiments.

3. *Cholinesterase Determinations*

Venous blood from the antebrachial cephalic vein was collected before injection of DFP and 24 hr. thereafter. The ChE activity was measured with the Warburg apparatus by the method of Ammon (2) and Odom *et al.* (33) using ACh as substrate.

ChE activity was taken as the volume of carbon dioxide in cu. mm. produced in 60 min. by 1 ml. of undiluted enzyme preparation. Decrease in ChE activity after DFP injection was expressed as the percentage of the preinjection level. Compensation was made for autohydrolysis of the ACh and for barometric and temperature variations.

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* *Cats were used in all experiments.*

** *DFP was obtained from the Army Chemical Center, Maryland, U.S.A.*

*** *Sodium fluoroacetate was obtained from Dr. E. S. G. Barron through Dr. K. A. C. Elliott.*

The red cell ChE activity reflects better than does the plasma the activity of the brain ChE. At least this is true for the rat, rabbit, monkey, dog, and man (22, 24, 31) and probably also for the cat. Difficulty was experienced in the measurement of the cat red cell ChE because of the very low values found even before the injection of DFP. A suitable method was developed and was used in the later experiments.

The red cells were washed twice in bicarbonate–Ringer and then hemolyzed in 0.024 *M* sodium bicarbonate in a volume twice that of the whole blood from which they were taken. The "ghosts" were then homogenized by the instrument of Potter and Elvehjem (35) and 0.5 ml. of the homogenate was placed in the side arm of the vessel. The procedure was then the same as for plasma.

4. *Acetylcholine Assays*

The presence of ACh in the CSF was determined with the isolated ventricle of *Venus mercenaria* according to the method of Wait (39) and Tower and McEachern (38). Samples of CSF showing ACh were submitted to hydrolysis and tested again as further identification. To the CSF was added 0.1 *N* sodium hydroxide, the mixture was boiled momentarily, and then neutralized with 0.1 *N* hydrochloric acid.

ACh detectable in the original CSF varied from 0.4 to 10 μgm. %. Clam heart preparations sensitive to 3 μgm. % or less were used. CSF samples were preserved by the method of Tower (36).

5. *Metrazol and Thujone Seizures*

Metrazol (pentamethylentetrazol) and thujone were used to produce seizures by intravenous injection. An ampoule of 10% *metrazol* was diluted one-third in water, and an amount containing 17 mgm. per kgm. was injected rapidly. *Thujone* was prepared as a 1% emulsion in 6% gum acacia, and 0.7 ml. per kgm. of the emulsion was injected rapidly (21). Both drugs in these quantities produced violent convulsions lasting 15 to 45 sec. Thujone was also given following a previous injection of DFP. CSF for ACh determinations was removed 15 to 50 min. following the onset of seizures.

6. *Electric Shock*

Single a-c. shocks of 130 v., 0.3 sec., were given through bitemporal electrodes with a Rahm electroconvulsive therapy machine; d-c. shocks of 85 to 145 v., 2 to 9 sec., were given to other animals. CSF for ACh determinations was removed two to three hours following the shocks.

7. *Electroencephalograms*

Cortical potentials were recorded in two of the intracarotid DFP experiments. The cat was put to sleep with pentothal. Four steel electrodes, made from Kirschner wire, were sharpened at one end and insulated with

Tygon to the tip and were placed symmetrically into the skull. Bipolar recordings were taken with an ink-writing electroencephalograph whose frequency response was 0.5 to 40 cycles between the 30% attenuation points and whose over-all linear amplitude was 1.5 cm.

Results

1. *Carotid Artery Injections of DFP (see Table I)*

During the injection there was a series of prolonged twitches of the musculature about the incision. In the control only very slight twitches were seen. In two of the experiments the artery dilated to almost twice its size during the course of the injection. Salivation began a few minutes later, and, when the dose was relatively high, it lasted for several hours. In all but one there was a pronounced constriction of the pupil on the side of the injection. This ipsilateral myosis lasted from hours to days. Intermittent tremor of the limbs and trunk appeared half an hour after the injection and lasted for hours.

TABLE I

INTRACAROTID INJECTIONS OF DFP

No.	DFP, mgm./kgm.	Immediate effects		Later effects			% Normal ChE	
		Dilat. of artery	Muscle spasm	Ipsilateral salivation	Ipsilaterai myosis and duration	Ipsilateral tremor	Plasma	RBC
8	0	No	Slight	No	No	No	100	—
2	0.9	No	No	Yes	18 days	No	24	—
4	0.9	Yes	Yes	No	No	Yes	35	32
5	1.35	Yes	Yes	Slight	24 hr.	Yes	14	15
6	1.35	No	Yes	Slight	2 hr.	Yes	15	20
7	1.63	No	Yes	Profuse	6 days	Yes	13	0
3*	1.80	No	Yes	Profuse	Intense	Yes	17	4

* *The animal in this experiment died in 24 hr.*

Plasma ChE levels were low 24 hr. after DFP, in proportion to the amount injected (Table I). Red cell ChE levels were not measured in all experiments but they too were reduced considerably after DFP injection. The drug had therefore passed through the brain and into the systemic circulation.

In two of the experiments electroencephalograms (E.E.G.'s) were made before and 30 hr. after the injection. In one, 1.63 mgm. per kgm. of DFP had been given and there were E.E.G. abnormalities consisting of rapid sharp waves that came only from the side of the injection (Fig. 1). The other animal, which had received 1.35 mgm. per kgm. of DFP, showed no E.E.G. changes at the time the record was made. No clinical seizures were observed at any time.

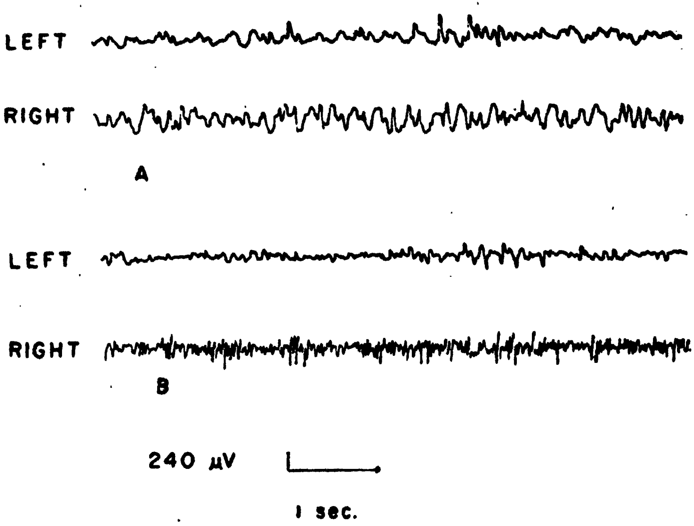

Fig. 1. *Electroencephalographic changes following injection of DFP. Experiment DFP-7, 1.63 mgm. per kgm. of DFP injected into right carotid artery. A, preinjection control. B, 30 hr. after injection.*

2. *Intracisternal Injections of DFP*

When a minimal dose was injected there was no immediate response. With a larger dose the depth of anesthesia was increased, as shown by loss of muscular tone and lack of response to stimuli. The duration of anesthesia was about three times that to be expected from the amount of pentothal used. This was very striking. Increased rate and depth of respiration followed within a few minutes in five of these experiments. Salivation, lachrymation, nystagmoid movements of the eyes, and twitching of the whiskers, all of short duration, were frequently seen in the first 10 min. Obviously the drug was affecting the brain-stem nuclei. When death occurred it was from respiratory failure. This was attributed to local action of the drug on the medulla.

Muscular tremor was noted within two hours of injection and lasted intermittently for one or two hours. The tremor resembled fasciculation.

When a forelimb of a conscious or even lightly anesthetized cat is pinched with forceps, or when the cornea is gently touched, there is usually a quick response. A diminished or absent response to this stimulation is described as sensory loss in Table II. It was a constant finding that persisted for many hours and sometimes for days.

Waddling gait with weakness of the hind limbs was observed in nine of the experiments after a sublethal dose of DFP. The animals showed ataxia upon awakening, and it was attributed to the anesthetic. However, the ataxia persisted for twice the usual time when DFP had been injected.

Plasma and red cell ChE dropped sharply from the preinjection levels and indicated that the DFP was absorbed into the general circulation.

TABLE II

INTRACISTERNAL INJECTIONS OF DFP

Summary of 23 experiments

No. of expts.	DFP, mgm./kgm.	Early effects (within 10 min.)	Later effects (within two hours)	% Normal ChE	
				Plasma	RBC
6	0-0.012	None	None	90-100	—
13	0.086-0.240	Deepened anesthesia Hyperpnea Salivation Lachrymation Nystagmoid eye movements Twitching of whiskers	Tremors Sensory loss Weakness of hind limbs Waddling gait	22-51	46-91
4	0.214-0.272	Deepened anesthesia Apnea and death by respiratory failure		8-24	—

3. *Intracisternal Injections of Sodium Fluoroacetate (see Table III)*

Increasing doses (up to 0.005 mgm. per kgm.) were given in six experiments but produced no definite effects. In the next 17 experiments 0.028 to 0.279 mgm. per kgm. was injected and definite effects were observed. These were of three main types and are here described as "scratching seizures", "scissors spasms" of the forelimbs, and myoclonic jerks. Usually two of the three appeared in the same animal and would continue for some hours until the animal was exhausted or the convulsions were stopped with nembutal.

"Scratching seizures" appeared in 11 of the 17 experiments listed in Table III, and the average time of onset was *three-quarters of an hour* after injection.

TABLE III

INTRACISTERNAL INJECTION OF SODIUM FLUOROACETATE

Summary of 23 experiments

No. of expts.	Sodium fluoroacetate, mgm./kgm.	Effects noted	Average time of onset after cisternal puncture
6	0.00002-0.005	Occasional twitch (not attributed to sodium fluoroacetate)	¼ hr.
17	0.028-0.270	1. "Scratching seizures" 2. "Scissors spasms" of forepaws 3. Myoclonic jerks	¾ hr. 1 hr. 2 hr.

At first the animal would briefly and accurately scratch the shaved area on the back of its neck with one or the other hind foot. This was repeated with increasing frequency, more and more violently, and with greater amplitude but less accuracy. A sustained opisthotonic or lateral contortion of the trunk accompanied the scratching. Finally the movements became almost continuous, with perhaps a half-minute interval occurring every three minutes.

By the "scissors spasm" is meant a tonic crossed adduction spasm of the forelegs, usually with the neck forcibly flexed. This lasted for several seconds and frequently heralded a scratching seizure. The "scissors spasm" appeared on an average one hour after cisternal injection and was seen in 12 of the 17 experiments.

Myoclonic jerks of the extremities or trunk appeared in over half of the animals, usually after a latent period of two hours. Sometimes they appeared after the scratching seizures or scissors spasms were well established, and then the myoclonic jerks gradually replaced the other phenomena as the chief manifestation. In other animals myoclonic jerks alone were seen. Death, when it occurred, was by respiratory arrest.

Sodium fluoroacetate (0.090 to 0.270 mgm. per kgm.) was also given *by vein* to five cats. After a latent period of *two to three hours*, either myoclonic jerks or tonic seizures were seen, but none like the scissors spasm or the scratching seizures. The earlier occurrence of seizures following intracisternal injection, and also their different character, are worthy of note.

The peculiar scratching seizures were investigated further. Some cats with shaved necks tend to scratch themselves mildly and fleetingly, especially upon awakening from the effects of an anesthetic. This may occur irrespective of what has been injected into the cisterna. The scratching seizures observed by us, however, were characterized by their violence, their duration, and their apparently involuntary nature. In some cases the animals did not appear to be conscious. In one experiment, when the scratching seizures were well under way, the skin of the neck in the shaved area was infiltrated with 1% novocaine. This had no effect upon the seizures.

The convulsions induced by fluoroacetate were easily arrested by intraperitoneal nembutal (22 mgm. per kgm.), which took effect after about 10 min. The animals were completely recovered by the next day.

Cisternal fluid was examined before and two to five hours after the injections in five experiments but no pleocytosis was found.

4. *Presence of Acetylcholine in Cerebrospinal Fluid*

Fifty-two samples of CSF were withdrawn from the cisterna magna of 20 cats before and/or after all types of procedures, and they were tested for ACh. Of these, 12 were controls, and the remaining 40 samples were taken at various times following the intracisternal or intravenous injection of the several drugs, and after electric shock. Amongst the controls, 0.4 μgm. % of ACh was found in two samples.

After intracisternal injections of DFP in high doses ACh was detected in the CSF in two out of five experiments. In one of these the original sample contained no ACh; four hours after injection (0.220 mgm. per kgm), 0.4

μgm. % of ACh was found. In the other experiment the control sample contained 0.4 μgm. % ACh; 15 min. after injection (0.261 mgm. per kgm.) the amount had increased to 1.0 μgm. %.

In seven experiments convulsant doses of sodium fluoroacetate were injected into the cisterna magna. CSF taken during the height of the seizures showed ACh in only one, and the control sample contained the same amount (0.4 μgm. %).

When a lethal dose of DFP by intravenous injection (5 mgm. per kgm.) was given, 3.0 μgm. % ACh was found in the CSF 10 min. later. Sublethal doses in four experiments produced no detectable ACh in samples drawn 30 to 60 min. later.

ACh was not found in any of three experiments where sodium fluoroacetate was injected by vein in doses that produced seizures (0.200 to 0.270 mgm. per kgm.). Neither did convulsions produced by metrazol (five experiments) or thujone (five experiments) increase ACh in the CSF to amounts that could be detected.

In four experiments convulsions were induced with thujone about one hour after DFP was injected intravenously, both drugs in amounts that had not produced ACh in detectable quantities in the CSF in previous experiments. In three of these experiments ACh was found to be present in the CSF 15 to 30 min. after the convulsion, in quantities ranging from 0.4 to 2.0 μgm. %.

Finally, ACh was not found in six experiments when seizures were produced by electric shock. Perhaps the presence of an anticholinesterase is necessary.

In these experiments the presence of ACh was detected only when the threshold of sensitivity of the clam heart happened to be at the lowest in the series (0.4 μgm. %). When there was no response to a particular sample of CSF the ACh content was reported negative. There may have been ACh present, however, in amounts that could only be detected by a more sensitive test preparation.

Discussion

It is known that brain tissue can synthesize ACh through its choline-acetylase enzyme system (30). ACh, or the anticholinesterase drugs eserine or neostigmine, can produce electrical evidence when applied locally to brain cortex in suitable concentration (8, 25, 27). ACh and eserine can produce somatic effects when applied to the brain in various ways (25, 26). ACh has also been recovered from the CSF of animals and humans after convulsive seizures (12, 37). Atropine sometimes prevents these central cholinergic phenomena, but its inability to do so should not rule out a basic cholinergic mechanism.

Injections of DFP

DFP (18, 19, 40) when injected in various ways does affect the electroencephalogram. But it seems clear that, when injected in sublethal quantities into the carotid artery or the cisterna magna of cats, it does not produce a lasting convulsive effect.

Salivation and myosis following intracarotid injection of DFP in our experiments was probably a peripheral effect in the area of distribution of the external carotid artery. Brassfield *et al.* (4) report continuous salivation from injection of DFP into the isolated arterial supply of the submaxillary gland. Ipsilateral myosis was also obtained by Himwich and Freedman (20) following intracarotid injection.

The early effects following intracisternal injection of DFP are probably due to local action on centers in the medulla and pons near to the floor of the fourth ventricle. A sluggish response to pain after DFP has been described by Modell *et al.* (28) who injected this substance intravenously in cats. In our experiments the sensory loss was confined to the forelimbs and the cornea. We have no explanation for this, nor for the waddling gait and weakness of the hind limbs. They appear to be specific effects, however, and did not occur with control injections.

The strange thing is that DFP does not produce a greater effect on the brain. It is a lipoid-soluble substance, yet in our experiments, when it was injected so as to place it in close proximity to the brain it did not produce the results that might be expected. In fact, it passed through the brain and, in proportion to the injected amount, produced a lowering of plasma and red cell ChE in the systemic circulation.

Injections of Sodium Fluoroacetate

The action of this drug shows marked species variability (9). In dogs and guinea pigs it has an effect primarily on the central nervous system; in cats and rhesus monkeys it produces both a cardiac and a nervous system response; in rabbits and goats it affects chiefly the heart. By administration into the cisterna magna of cats we attempted to produce a more selective action on the brain.

Chenoweth and St. John (10, 11) obtained wave and spike forms of E.E.G. activity resembling that of petit mal when they injected fluoroacetate directly into the cerebrum of the rabbit through a burr hole in the skull. They infer (11) that fluoroacetate is incapable of traversing the blood–brain barrier in this animal. Disturbance of electrical activity in the brain appeared with a much shorter latent period following intracerebral injection. This agrees with the relatively short latent period (three-quarters of an hour) following intracisternal injection in our own experiments. When the drug is given intravenously there is a latent period of two to three hours before convulsant effects appear. The still considerable period that constantly precedes the cerebral action of fluoroacetate, even when given intracisternally, is unexplained.

Acetylcholine in Cerebrospinal Fluid

All available reports that ACh may be found in the CSF, together with our own results, are summarized in Table IV. Here it is seen that as more sensitive methods are used ACh is detected more frequently. It is possible

TABLE IV

ACETYLCHOLINE IN THE CEREBROSPINAL FLUID

Summary of reports in the literature

Author	Subject	Procedure	ACh found, µgm. %	Test object
Dikshit (15)	Cat	Vagal stimulation	1.0	Frog heart
Feldberg and Schriever (17)	Dog	1. Injection of eserine 2. Injection of adrenalin 3. Asphyxia	1.5 2.5 5.0	Cat's B.P. and frog heart
Adam *et al.* (1)	Cat Dog	Perfusion of ventricular system with eserinized fluid Stimulation of hypothalamus	Positive; amt. not stated	Not stated
Chang *et al.* (7)	Dog	Vagal stimulation in eserinized animal	2.0–8.0	Leech muscle
Bornstein (3)	Cat Dog	Experimental brain trauma	2.7–9.0	Frog rectus abdominis
Tower (37)	Dog	Canine epilepsy induced by agenized flour	1.0 (2 expts.)	Clam heart
Nochimowski (32)	Human	Misc. neurological and psychiatric cases	10.0 (23 cases)	Leech muscle
Brecht (5)	Human	Meningitis; epilepsy	10^{-4}–2×10^{-2}	Frog lung
Brecht and Kummer (6)	Human	Psychiatric cases (schizophrenia and depression states)	10^{-6}–10^{-1}	Frog lung
Cone, Tower, and McEachern (12)	Human	1. Epilepsy	0.1–5.0 (44 out of 56)	Clam heart
		2. Brain trauma	0.5–100 + (7 out of 20)	
		3. Electric shock	0.2 and 2.0 (2 out of 6)	
Kelen and McEachern	Cat	1. Controls	0.4 (2 out of 12)	Clam heart
		2. Intravenous DFP	3.0 (1 out of 5)	
		3. Intracisternal DFP	0.4–1.0 (2 out of 5)	
		4. Intracisternal fluoroacetate	0.4 (1 out of 7)	
		5. Intravenous metrazol or thujone or electric shock	0.0 (15 expts.)	

that traces of ACh are always present in the CSF, even under normal circumstances, and that its significance will depend upon the *amount* found. Quantitative determination, by a suitably sensitive test object, may reveal that the amount of ACh varies with the kind of experimental procedure in animals, or with the nature of the illness in man. The clam heart (38, 39) will detect 0.04 µgm. % of ACh in the CSF in its best season. The positive results obtained by Tower and McEachern were largely in the range of 0.5 to 2.0 µgm. %, which was just beyond the threshold of many of our test objects during the summer when these experiments were carried out. The frog lung preparation (5, 13, 14), which requires 8 to 10 ml. of fluid to be tested, will detect as little as 0.000001 μgm. % in the winter months. The latter method is however, very delicate and time-consuming. More studies are needed to establish the role of ACh in the production of seizures. Its appearance in CSF may only be an echo of events.

The effects of DFP are believed to be due to its activity as an anticholinesterase. Previous workers (7, 17) have found ACh in the CSF following the intravenous injection of eserine. Hence it is surprising that ACh is not found until lethal amounts of the more potent anticholinesterase, DFP, are given. It may be that too little DFP is taken up by the brain to reduce the brain cholinesterase to the necessary level.

These studies neither refute nor uphold the hypothesis that ACh has a key role in the production of seizures. Further analysis of the biochemical mechanism of seizures should be carried out on brain tissue itself.

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 27, SEC. E. JUNE, 1949 NUMBER 3

THE DETERMINATION OF PHOSPHOLIPID PHOSPHORUS[1]

BY J. M. R. BEVERIDGE AND S. E. JOHNSON

Abstract

A simple accurate method for the determination of phospholipid phosphorus is described. Conditions for the digestion of phospholipid have been developed permitting the utilization of a colorimetric procedure for inorganic phosphorus that is characterized by excellent color stability and strict adherence to Beer's law over a range of 0 to 65 μgm. of phosphorus.

Introduction

The existence of such a large number of modifications for the determination of phosphorus by the "molybdenum blue" procedure (Woods and Mellon (5)) attests to the fact that these methods have not been entirely satisfactory.

Following a trial of one of these procedures (King (3)) with results that were undesirable (cf. also Allen (1)), a review of the literature was made to find a suitable method or one that could be adapted readily to the requirements of the work to be performed. These were:

1. The rapid digestion of organic matter without loss of phosphorus.
2. A stability of color that would permit the comparison of a large number of samples at one time.
3. Adherence to Beer's law of the relationship between phosphorus concentration and color density over a range of 5 to 65 μgm. of phosphorus.
4. A degree of error not more than approximately 1% in the colorimetric procedure.

Experimental

After a survey of a number of the modifications of the molybdenum blue method, it was decided that the procedure for inorganic phosphorus described by Boltz and Mellon (2) appeared to hold most promise for adaptation to the determination of phospholipid phosphorus. These workers showed that the blue color obtained when molybdic acid itself is reduced differs from that obtained from the reduction of the phosphoric – molybdic acid complex. The former absorbs light most strongly in the region of 630 to 650 mμ, whereas the latter color, termed heteropoly blue to distinguish it from molybdenum blue, has a characteristic absorption band at 820 to 830 mμ.

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[1] *Manuscript received February 3, 1949.*

A contribution from the Department of Pathological Chemistry, Faculty of Medicine, University of Western Ontario, London, Ont., with financial assistance from the Life Insurance Medical Research Fund, New York, U.S.A.

They stated that the color was stable for 12 hr. and that Beer's law held over the range 0 to 1.5 parts per million (0 to 75 μgm.) of phosphorus. An error of 2% was considered to be negligible.

These statements were confirmed and, since they were not accompanied by illustrative data in the original paper, the results of the present work are given here. Fig. 1 shows the relationship between color density and phosphorus concentration. Table I reveals that there is excellent color stability up to at least 23 hr., beyond which time it was not tested. An examination of the data in the table also shows that the percentage error is of the order of only 1% or less.

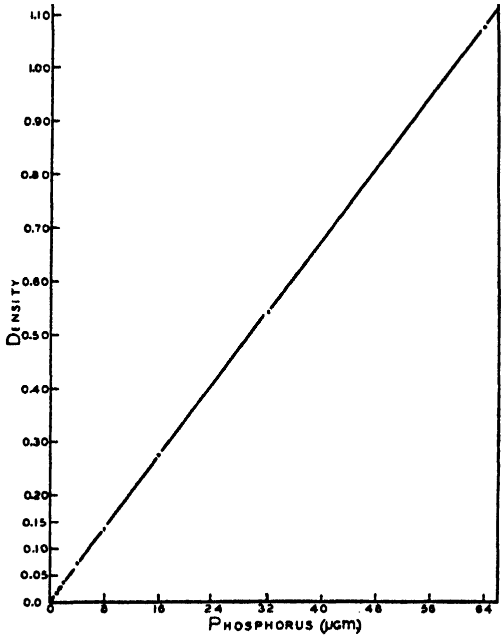

FIG. 1. *Relationship of phosphorus concentration to density of "heteropoly" blue color.*

TABLE I

STABILITY OF HETEROPOLY BLUE COLOR

Sample	Density	
	1 Hr.	23 Hr.
1	0.338	0.339
2	0.343	0.341
3	0.338	0.337
4	0.342	0.340
5	0.336	0.335
6	0.335	0.333
7	0.337	0.335
8	0.342	0.339

The problem, therefore, resolved itself into the elaboration of adequate conditions for the digestion of the phospholipid that would permit the use of this excellent colorimetric procedure. Since the color development took place in 1*N* sulphuric acid, it was obvious that the digestion had to be carried out in this medium. In the digestion of organic matter for the determination of phosphorus, the following points must be taken into consideration.

1. The digestion should be rapid and complete.

2. There must be no significant loss of sulphuric acid since this would affect markedly subsequent color development by permitting the reduction of molybdic acid itself with the formation of molybdenum blue.

3. There must be no loss of phosphoric acid. The possibility of error being introduced by this factor is very real since phosphoric acid is volatile at a temperature of 213° C., whereas concentrated sulphuric acid boils at 330° C.

4. There must be no conversion of orthophosphoric acid to meta- and pyrophosphoric acids or, if there is any such conversion, it must be reversed since the latter compounds do not react to give a blue color.

Various combinations of potassium sulphate, copper sulphate, selenium dioxide, and mercuric oxide were used as catalysts to speed the oxidation of the total organic matter by heating with 1 ml. of concentrated sulphuric acid. The digestion time was reduced to between 5 and 10 min. but all these catalysts were found to interfere to some degree with subsequent color development. Consequently, attempts to utilize them were abandoned.

Finally, the following procedure was adopted. The phospholipids were precipitated according to the method of Sinclair and Dolan (4) in a Pyrex test tube about 15 cm. in length, 1.4 cm. in diameter, and about 20 ml. in capacity. Any organic solvent remaining after the tubes were drained was completely driven off. One ml. of concentrated sulphuric acid was added and the tube heated over the low flame of a microburner for about five minutes. Two drops of 30% hydrogen peroxide were then added to the charred mixture, shaking thoroughly between each drop. This treatment practically completed the oxidation. Heating was continued a further 10 min. to ensure completion of oxidation and destruction of excess hydrogen peroxide. If any of the latter substance is allowed to persist, color development is seriously inhibited.

In order to determine the minimal length of time necessary for complete combustion and to ascertain whether or not any significant loss of sulphuric and phosphoric acids occurred during digestion, the following experiment was performed. Samples of lipid extract were digested in the usual way for periods up to 60 min. Glass funnels with stems drawn to a fine tip and sealed with a drop of water were placed in a number of the digestion tubes to prevent loss of sulphuric and phosphoric acids. The results are shown in Table II and indicate that a 15 min. digestion time is adequate. Since there is no difference in the results derived from the series with and without funnels, it may be concluded that no significant loss of either sulphuric or phosphoric acid occurs under the conditions employed. Titrimetric data for the former acid support this conclusion.

TABLE II

CONDITIONS FOR DIGESTION OF PHOSPHOLIPID

	Time	
	15 min.	60 min.
	Density	
With funnel	0.338	0.336
Without funnel	0.342	0.339
Diluted digest boiled	0.342	

The possibility that the conditions of the combustion effected a conversion of orthophosphoric acid to the meta- and pyro-forms was investigated by boiling the digested sample with 24 ml. of water for about 10 min. This treatment reconverts any of the latter compounds to orthophosphoric acid. The data obtained are shown also in Table II. If meta- and pyrophosphoric acids had been present, the unboiled samples would have given lower results since these forms do not react with molybdic acid to yield a compound that, on reduction, will form a blue color. The close similarity between the amounts of phosphorus found in the boiled and unboiled samples precludes the existence of any such conversion under these conditions.

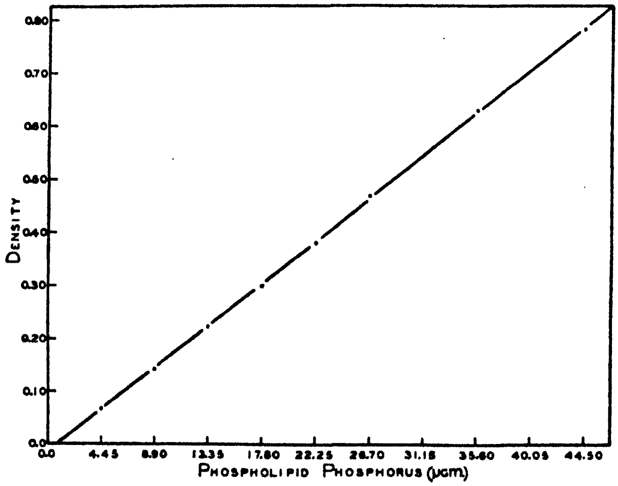

FIG. 2. *Effect of sample size on phospholipid determination.*

In view of the marked sensitivity of the colorimetric procedure, the question arose regarding the minimal aliquot of mixed lipids that could be used and that would still permit a reasonable degree of accuracy in the determination of phospholipids. This point was investigated by the precipitation of the phospholipids in varying aliquots of a crude lipid extract according to the method of Sinclair and Dolan (4). The precipitates were digested as previously described and total phosphorus determined. The results, in Fig. 2, reveal that amounts of phospholipid as low as 0.1 mgm., equivalent to approximately 4 μgm., may be utilized without sacrifice of accuracy.

The method in detail is given herewith.

1. Add 1* ml. of concentrated sulphuric acid to the phospholipid sample in a Pyrex test tube.

2. Heat for five minutes over the low flame of a microburner; add two drops of 30% hydrogen peroxide (low in phosphorus), shaking between the addition of the drops. Heat 10 min. longer.

3. Cool; add 9* ml. of distilled water and transfer to a 50 ml. volumetric flask with the aid of 15* ml. of distilled water.

4. Add 20* ml. of freshly prepared molybdate – hydrazine sulphate reagent** from a rapid delivery pipette.

5. Make the solution up to volume with distilled water, mix, loosen stopper, and place the flask in a boiling water bath for 10 min.

6. Remove and cool the flask rapidly by placing it in a cold water bath until the level of the liquid again reaches the mark.

7. Measure the color density within 24 hr. at 830 mμ.

Blank determinations were performed on the reagents using 1 ml. of concentrated sulphuric acid and two drops of 30% hydrogen peroxide. The amount of color obtained was equivalent to only 0.26 μgm. of phosphorus. It may be noted that this amount represents about 1% at a level of 20 μgm. of phosphorus in the test sample.

This method affords an accurate, rapid determination of phospholipid phosphorus and, because of the color stability, it lends itself admirably to the performance of a large number of analyses at a time.

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* *These volumes are not critical.*

** *This reagent was originally described by Boltz and Mellon as being prepared by combining 25 ml. 2.5% (W/V) sodium molybdate dihydrate in 10N sulphuric acid with 10 ml. of 0.15% (W/V) hydrazine sulphate and making up to 100 ml. with distilled water. The amount of acid contained in 20 ml. of this reagent made the final solution 1N with respect to sulphuric acid.*

Since the concentrated sulphuric acid used in the digestion process contained about 35 m. e., the sodium molybdate had to be dissolved in more dilute acid. A concentration of 3N sulphuric acid yielded the necessary m. e. to make the final solution 1N with respect to acid.

AGGLUTINATION OF ERYTHROCYTES CONTAINING SULPHAEMOGLOBIN – FURTHER EVIDENCE OF SPECIFIC AGGREGATION[1]

By Kenneth W. McKerns and Orville F. Denstedt

Abstract

By sulphuration of red blood cells with hydrogen sulphide, up to 75% of the haemoglobin can be converted to sulphaemoglobin without seriously impairing the osmotic stability or the agglutinability of the cells with various antisera with exception of anti-Rh. Unlike the A, B, M, and N agglutinogens, the Rh factor is labile to sulphuration and numerous other treatments. Sulphurated cells, by their size and color, can be distinguished from normal cells under the microscope. This feature has been used to confirm Marrack's hypothesis that the aggregation phase of the agglutination reaction is specific.

The agglutination of erythrocytes, bacteria, and colloidal antigens by specific antisera is commonly regarded as being a two stage process. In the first stage, antibody of the serum combines with antigen on the cells or particles, producing a state of "sensitization." In the second, or "aggregation" stage, the cells or particles cohere and precipitate out in clumps. Sensitization is highly specific, but whether the aggregation process also is specific is more difficult to prove. Bordet (3) maintained that the latter is nonspecific; that combination of antigen with antibody so alters the properties of the cells or particles as to render them susceptible to flocculation by the electrolyte in the surrounding medium.

Several years later Marrack (16) advanced the theory of specific aggregation, frequently referred to as the "framework" or "lattice" hypothesis, which implies that homologous antibodies constitute the links that hold aggregated cells or particles together. Thus, if cells, differing in antigen composition, be mixed and agglutinated simultaneously with their respective homologous antisera, the aggregates according to Bordet, should consist of a mixture, whereas according to Marrack each type of cell should form separate aggregates.

The preponderance of experimental evidence supports Marrack's hypothesis. Heidelberger and Kendall (8-12) precipitated polysaccharide, azoprotein, and other protein antigens with immune serum, and showed that aggregation is specific. Topley, Wilson, and Duncan (27) used a mixture of two species of bacteria to demonstrate that simultaneous agglutination with the respective homologous antisera caused the organisms to form separate clumps. Wiener and Herman (29) also obtained discrete clumps with a mixture of Kline antigen and human red corpuscles. The classic studies of Pauling and his associates (20-25) with coupled azoprotein compounds provide especially strong evidence in support of Marrack's hypothesis. On the other hand, Abramson (1) obtained both homogeneous and heterogeneous clumps with a mixture of sheep

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[1] *Manuscript received February 1, 1949.*
Contribution from the Department of Biochemistry, McGill University, Montreal, Que.

red cells and Friedlander bacilli. Likewise, Hooker and Boyd (13) mixed mammalian and avian red corpuscles with the respective antisera and obtained heterogeneous clumps suggestive of nonspecific aggregation. Later, however, Abramson and Boyd (2) repeated the experiments with partially haemolyzed red cells and observed a tendency towards specific aggregation.

Duncan (5), in some measure reconciles the two hypotheses. On agglutinating mixtures of flagellated and nonflagellated bacteria with immune sera, he obtained conclusive evidence that both stages of agglutination were specific. In precipitin test with mixtures of yeast polysaccharide and crystalline albumin, however, there was evidence of both modes of aggregation, the primary mechanism being specific and followed by a secondary nonspecific aggregation.

The kinetics of agglutination based on the Bordet hypothesis has been studied by Teorell (26) and by Morales (17), who have derived equations on the assumption of a univalent type of antibody and a polyvalent antigen. Analogous studies based on the specific aggregation hypothesis have been reported by Heidelberger and others (7). The mathematical treatments have been reviewed by Boyd (4), while the fundamental mechanism of the reaction and the nature of the forces involved have been discussed by Pauling (18, 19).

Further confirmation of Marrack's hypothesis has now been obtained in our laboratory by use of a mixture of normal and sulphurated erythrocytes. As shown below, red cells can be sulphurated *in vitro* to a high content of sulphaemoglobin without altering their agglutinability. The sulphurated cells are pale green and are slightly larger in diameter than normal cells. On agglutination they undergo less crenation and shrinkage in diameter than normal cells, and thus are readily distinguishable from the latter in an agglutinated mixture. The experiments here reported concern the influence of sulphuration on agglutinability of human red corpuscles and use of sulphurated cells in confirming the specific aggregation hypothesis.

Experimental

Preparation of Sulphurated Red Cells

A suspension (about 5%) of human red cells from a few drops of blood was made with isotonic phosphate buffer* of pH 7.35 in a small serological test tube (10 × 75 mm.). The air in the tube was displaced with hydrogen sulphide, and the tube was stoppered and slowly and repeatedly inverted. Addition of hydrogen sulphide and mixing were repeated two or three times depending on the degree of sulphuration desired. After standing for 20 min. at room temperature with occasional mixing, the red cells assumed a deep greenish color. To remove residual hydrogen sulphide the tube was centrifuged lightly, the supernatant fluid removed by aspiration, and the cells washed with the phosphate buffer solution. Washing was repeated until the supernatant gave a negative test with lead acetate. The proportion of sulphaemoglobin to haemoglobin in the cells was determined by the method of Evelyn and Malloy (6). No attempt was made to distinguish between sulphaemoglobin and choleglobin (15), which, if present, would be measured as sulphaemoglobin. To ensure ready differentiation of the sulphurated and normal cells in agglutination experiments, the sulphaemoglobin content of the cells should be between 50 and 75% of the total pigment.

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* *The buffer mixture was prepared by mixing 20 ml. of 4.17% sodium dihydrogen phosphate monohydrate and 16.5 ml. of 0.302 N sodium hydroxide.*

Stability and Agglutinability of Sulphurated Red Cells

It is important in agglutination studies that the stability and agglutinability of the cells should not be greatly altered by sulphuration. The following tests were performed to ascertain the effect of the treatment on these properties.

Stability

One ml. of lightly packed washed cells was suspended in 2 ml. of isotonic phosphate buffer (pH 7.35); 0.5 ml. portions of the suspension were dispensed into serological tubes. Hydrogen sulphide was introduced into the space above the liquid and the tubes were stoppered and agitated gently for 20 min. The excess hydrogen sulphide was then removed with the aid of an aspirating tube.

Osmotic stability was tested by the fragility method of Waugh and Asherman (28), who recommend the use of a buffered saline (Simmel's) in place of simple sodium chloride solutions.

The sulphurated cell suspension was pipetted in 0.02 ml. portions into 15 ml. centrifuge tubes containing isotonic Simmel's solution and a range of hypotonic concentrations. After five minutes the tubes were centrifuged for five minutes. The supernatant solution was discarded and the cell sediment suspended in 10 ml. of $M/60$ phosphate buffer of pH 6.6. One drop of each of the following solutions: concentrated ammonium hydroxide, 20% potassium ferricyanide, and 10% potassium cyanide was added in that order. The concentration of cyanmethaemoglobin (cyanhaemochromogen) was determined by Evelyn's method (6), with a 540 mμ filter.

TABLE I

CELL HAEMOLYSIS AFTER FIVE MINUTES IN SIMMEL'S SOLUTION

Pigment as SHb, %	Dilution, % of isotonic				
	100	70	60	50	40
	Haemolysis, %				
0	0	0	0	0	81
35	0	0	0	0	84
45	0	0	0	11	85
75	0	0	10	54	95

It will be observed from Table I that the osmotic fragility of the cells to hypotonic saline solutions is not greatly increased by sulphuration *in vitro.* For the present purpose the slightly increased fragility is of no consequence, since cells containing from 50 to 60% of sulphaemoglobin are stable for several hours in saline even down to 60% of the isotonic concentration. As all the agglutination tests were carried out in isotonic medium no breakdown of the sulphurated cells occurred during the 15 min. period of the test.

Agglutinability

Two criteria were adopted to validate the use of sulphaemoglobin for labelling the cells: (1) comparison of sulphurated and normal cells in titer tests with a given potent antiserum and (2) comparison of the number of free cells after agglutination of the sulphurated and normal cells.

(1) Titer Tests

When the potency of various strong antisera, including anti-A, anti-B, anti-M, anti-N, anti-Rh_o (anti-D), and anti-Rh″ (anti-E), was tested with appropriate cells, both normal and sulphurated cells from a given donor were found to give the same titer values except when anti-Rh sera were used. The Rh agglutinogens were found to be labile to numerous types of treatment whereas the A, B, M, and N antigens are very stable. Thus a lower titer was obtained with the Rh antisera especially when the sulphaemoglobin level in the cells was more than 50%. Obviously there is no change in the potency of the serum but the impaired reactivity of the Rh antigen results in a lower titer value.

(2) Free-cell Counts

Fresh blood was drawn up to the 1.0 mark in a white-cell pipette, which then was filled to the 11 mark with isotonic phosphate buffer of pH 7.4. After the pipette was shaken, the contents were transferred to a serological tube and an equal amount of buffer was added. The contents were sulphurated and gently agitated mechanically for 20 min. Finally, excess hydrogen sulphide was removed. Colloidal sulphur frequently is formed during sulphuration but is of no consequence.

Duplicate cell suspensions were prepared from blood from various Group A individuals according to the procedure outlined. One sample from each pair was sulphurated and then all samples were agglutinated with potent anti-A serum and the unagglutinated cells were counted according to the following procedure.

Blood is drawn up to the 1.0 mark in a white-cell pipette, which then is filled to the 11 mark with agglutinating serum. The pipette is shaken and the contents transferred to a serological tube. The tubes are gently agitated four or five times during a two minute period and then centrifuged for exactly two minutes at 1500 r.p.m. After flicking the tube to dislodge the agglutinated cell sediment the sample is agitated mechanically for 40 min. at the rate of 230 vibrations per minute.

For counting the free cells, the larger clumps are permitted to settle for about three seconds and a sample of the supernatant fluid is transferred to the haemocytometer by means of a white-cell pipette filled to the 1.0 mark. The free cells in the entire field (144 large squares) are counted and the number of cells per cu. mm. calculated as follows:

$$\text{Free cells per cu. mm.} = \frac{\text{total count} \times 11 \times 22 \times 10}{9}$$

$$= \text{total cell count by } 269 \ .$$

Results:

(*a*) Reproducibility of free-cell count.

The following figures were obtained from four successive agglutination tests on Group A red cells containing 71% sulphaemoglobin: 19,900; 18,300; 20,200; 20,100. Mean 19,625.

(*b*) Comparison of free-cell counts on normal and sulphurated cells.

The cell counts given in Table II represent the means of duplicate counts. The cells were taken from different donors, all of group ARh$_0$. In all instances anti-A serum of 512 titer was used.

TABLE II

FREE-CELL COUNT BEFORE AND AFTER SULPHURATION

Subject	Cell specimens	SHb, %	Duration of agitation at 230 vibrations per min.	Free-cells per cu. mm.
I	Normal	0	55	14,200
	Sulphurated	60	55	15,000
II	Normal	0	40	21,000
	Sulphurated	75	40	22,600
III	Normal	0	30	39,900
	Sulphurated	67	30	38,000
IV	Normal	0	45	53,000
	Sulphurated	70	45	51,400

It is evident that the reproducibility of the counts is satisfactory and that the agglutinability of the cells is not altered by the sulphuration treatment.

Use of Sulphurated Red Cells for Testing Marrack's Hypothesis

Sulphurated red cells, which are thus shown to be comparable in stability and agglutinability with normal cells, can be used for testing the specificity of aggregation. The general procedure was as follows: light saline suspensions of normal and sulphurated human erythrocytes were adjusted by dilution so that the concentration of cells in all tubes was the same. To facilitate differentiation of the normal and sulphurated cells in the aggregates, the serum was diluted so as to favor the production of small rather than large clumps. One

PLATE I

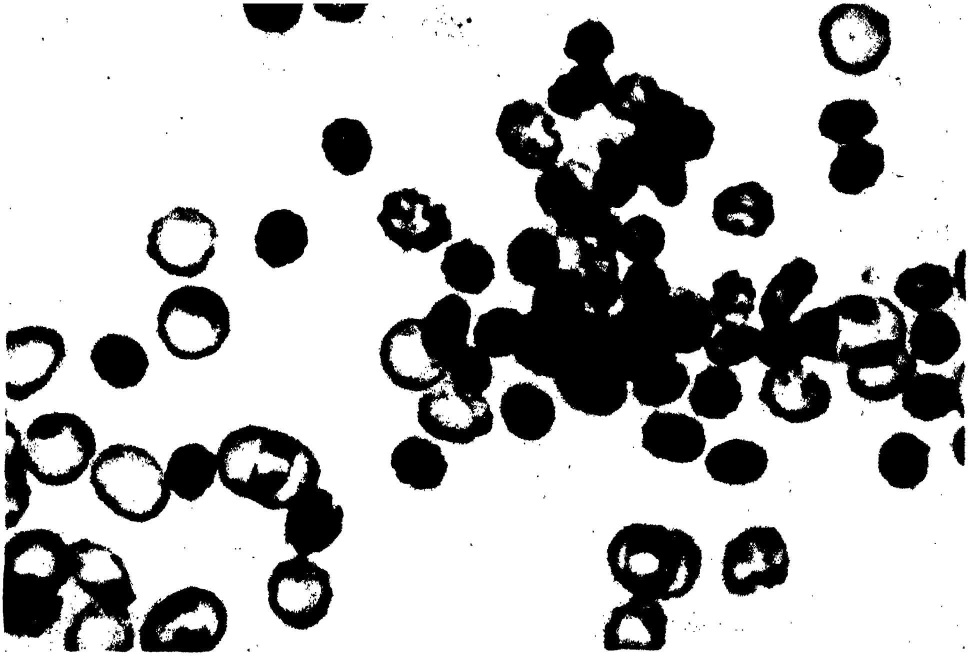

FIG. 1. *Appearance of normal and sulphurated red corpuscles in an agglutinated mixture.*

drop of each of the two cell suspensions was introduced into a serological tube with two drops of the appropriate antiserum or sera. The tube was centrifuged at low speed (about 1500 r.p.m.) for two minutes. The cell sediment was then broken into small clumps by flicking the tube. The microphotograph in Fig. 1 illustrates the difference in appearance of the normal and sulphurated erythrocytes. If a daylight lamp is used, the sulphurated cells appear larger, paler, and slightly greenish in color. Table III gives the results of a typical experiment.

TABLE III

SPECIFIC AGGLUTINATION WITH A AND B AGGLUTINOGENS

Blood group of red cells in mixture	Agglutinating serum	Composition of resulting clumps
A + A	Anti-A (titer 512)	Mixed *A*A
A + B	Anti-B (titer 512)	B only
A + B	Anti-A	A only
A + B	Anti-A + Anti-B	Separate *A* and B

Letters in italics designate sulphurated cells.

Analogous results were obtained with cell mixtures *B* + B, *B* + A, *M* + M, *M* + N, M + *N*, Rh_o + Rh$_o$, and Rh_o + Rh$''$ with the appropriate sera.

For the M- and N-tests, red cells of group BMRh$_o$ (50% SHb) and group ANRh$_o$ (46% SHb) were used with anti-M and anti-N sera (titer 32). The anti-M serum was absorbed with a light suspension of "N" cells, and the anti-N, with "M" cells, prior to using.

In the case of the Rh antigens the Rh$_o$ cells were sulphurated to a level of 45% SHb. Anti-Rh$_o$ (anti-D) and anti-Rh$''$ (anti-E) sera of titer 64 were used.

Discussion and Conclusions

From the foregoing results it is clear that sulphuration *in vitro* with hydrogen sulphide does not seriously alter the osmotic stability of human red cells, nor does it affect the agglutinability except with anti-Rh serum. The Rh antigen, being much more labile to various treatments than the A, B, M, or N antigens, is altered by sulphuration, especially when the sulphaemoglobin is about 50% of the total pigment. The A, B, M, and N antigens, on the other hand, are stable up to 75% sulphuration, which is the maximum level attainable by our method.

Examination of the clumps obtained by agglutinating mixtures of normal and sulphurated red cells of various types with appropriate antisera showed consistently that cells of different types formed discrete aggregates. In other words, both sensitizing and aggregating phases of the agglutination reaction appear to be specific, as postulated by Marrack and others.

Acknowledgments

This investigation, which is part of a larger study on agglutination, is being carried on with financial support from the National Research Council of Canada.

The writers are indebted to Dr. R. Denton, of the Children's Memorial Hospital, Montreal, for a supply of high-titer anti-A, anti-B, and anti-D serum, and to Dr. P. G. Weil, of the Royal Victoria Hospital, for anti-E serum.

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THE EFFECT OF ANTITOXIN ON EXPERIMENTAL INFECTION WITH *CLOSTRIDIUM NOVYI* (*OEDEMATIENS*) IN GUINEA PIGS, WITH A NOTE ON ACTIVE IMMUNIZATION[1]

By G. G. Waters and P. J. Moloney

Abstract

C. novyi antitoxin injected in divided doses (total 500 units) over a period of 12 days was more effective in saving guinea pigs inoculated with spores of *Clostridium novyi* than was the same total amount of antitoxin given in one dose. Of the infected animals, 60 to 80% could be saved by treatment with divided doses of antitoxin up to 12 hr. after inoculation with spores. If, however, treatment was deferred for 24 hr., none of the animals could be saved. Preliminary results on active immunization with *novyi* toxoid indicate that a relatively high titer of antitoxin (in the order of 1μ per ml.) is required to protect guinea pigs against inoculation with *novyi* spores suspended in calcium chloride solution.

Introduction

Treatment, with antitoxin, of experimental gas gangrene induced in guinea pigs by *Clostridium novyi*, has been reported by others. Gordon and McLeod (2) found that *novyi* antitoxin was not effective as a curative agent in the guinea pig inoculated in a crushed leg muscle with a 24 hr. broth culture of *C. novyi*. The maximum total amount of antitoxin per guinea pig that Gordon and McLeod used was 3600 units. This was injected in two doses at four and seven hours after inoculation.

Klose and Schröer (3) claimed that mice inoculated with a dilution of a 24 hr. broth culture of *C. novyi* mixed with mucin survived when *novyi* antitoxin was injected three hours after inoculation of the broth culture. The amount of antitoxin administered to each mouse was 0.7 ml. serum containing 56 units of *novyi* antitoxin.

Klose and Schröer probably injected the antitoxin subcutaneously since other antitoxins studied by them and reported in the same paper were injected subcutaneously. However, in the case of *novyi* antitoxin, the text indicating the route of injection reads P.O. indicating, no doubt, per os. This is probably a misprint since antitoxin per os would almost certainly not have been effective.

Evans (1) induced gas gangrene in the guinea pigs by injecting a suspension of washed young organisms into the thigh of the animal three hours after an injection of calcium chloride had been given. He found that, with *C. novyi* infection, protection from death could be achieved only by the intravenous injection of large doses of antitoxin (in the order of 2500 units per animal) in an early stage of the disease. Animals could be saved up to 16 hr. but not 24 hr. after inoculation.

[1] *Manuscript received in original form November 26, 1948, and, as revised, February 25, 1949.*

Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont.

In this communication we are reporting some experiments on the treatment with antitoxin of experimental *C. novyi* infection in guinea pigs; in addition, we are presenting some preliminary observations on active immunization of guinea pigs against *C. novyi* infection.

Methods

C. novyi infection was induced in guinea pigs by the intramuscular injection of spores of *C. novyi*.

The spore suspension used was prepared as follows: Kidney meat broth (4) was seeded with *C. novyi* N21D and incubated at 38° C. for five days. The supernatant broth culture was separated from the precipitate of ground meat and centrifuged. The supernatant, after centrifuging, was drawn off and discarded, and the deposited microorganisms were washed by mixing with sterile physiological saline and again centrifuging. This process of washing was repeated three times. The washed organisms were then resuspended in a volume of saline equal to that of the supernatant broth from which they came, and left in the cold room at 5° to 8° C. for two days, after which they were heated at 70° C. for 15 min. and then stored in the cold room at 5° to 8° C. This spore suspension showed no noticeable loss in killing power when tested on guinea pigs after storage for one year.

The spore suspension alone, when injected intramuscularly and without trauma, was not lethal for guinea pigs. Death, however, as a result of gas gangrene infection, could be induced, just as with tetanus spores, by injecting spore suspension diluted with calcium chloride solution. For this purpose we found that calcium chloride in a concentration of 8% was suitable.

TABLE I

INTRAMUSCULAR INJECTIONS INTO GUINEA PIGS OF SPORES OF *C. novyi* IN CALCIUM CHLORIDE SOLUTION

Dilution	Number of animals living	Number of animals dead	% Survival
1/10,000	0	33	0
1/100,000	5	31	14
1/1,000,000	33	1	97

In Table I there is given a summary of the effect of intramuscular injection of the spore suspension described above, in 8% calcium chloride solution. The guinea pigs used weighed about 500 gm. each and each animal was injected intramuscularly with 0.5 ml. of the diluted suspension.

It will be noted that all the guinea pigs that received the 1/10,000 dilution and most of those that received the 1/100,000 dilution died.

The *novyi* antitoxin used throughout was derived from the blood plasma of horses that had been hyperimmunized by injections of *novyi* toxin. The antitoxin in the plasma was purified and concentrated by a method involving peptic digestion.

Effect of Single and Multiple Doses of *novyi* Antitoxin

Preliminary experiments were carried out in which a single dose of antitoxin of varying strength, up to 500 units, was administered to guinea pigs at different times after inoculation with spores of *C. novyi.*

The administration of antitoxin rarely prevented death although the life of many of the animals was prolonged. As a result of this preliminary work, it was decided to set up an experiment in which the effect of antitoxin in single and divided doses might be compared. To this end, 180 guinea pigs of about 500 gm. each were injected in the thigh muscle with 0.5 ml. of a 1/10,000 dilution of spore suspension in 8% calcium chloride solution. At different times after the inoculation with spores, groups of the animals were injected intraperitoneally with *novyi* antitoxin. For example, with one group of 45 guinea pigs, each animal was injected with 500 units of antitoxin five hours after inoculation with spores; in a second group of 45 guinea pigs, each animal was injected, five hours after inoculation with spores, with 200 units of antitoxin followed by 50 units every 48 hr. until a total of 500 units had been injected.

TABLE II

CURATIVE ACTION OF ANTITOXIN FOLLOWING INJECTION OF SPORE SUSPENSION

Interval between inoculation of spores and administration of antitoxin (hours)	Survivals after:	
	500 units in one dose	200 units + 50 units × 6 over a period of 12 days
5	4/45 (9%)	27/45 (60%)
8	1/15 (7%)	9/15 (60%)
12	1/15 (7%)	12/15 (80%)
24	0/15 (0%)	0/15 (0%)

Results are summarized in Table II. In the table there is set forth the times of administration of the antitoxin following inoculation with spore suspension and the manner in which the antitoxin was given, that is, in single and divided doses, and also the number of guinea pigs in each group that survived.

It will be noted that 500 units of antitoxin injected in divided doses over a period of 12 days was more effective in saving the guinea pigs than was 500 units in one dose.

In order to obtain information regarding the antitoxin titers of the blood of guinea pigs that had been injected with spores of *C. novyi* followed by antitoxin, blood titrations were carried out on some of the guinea pigs of the group that were treated with antitoxin five hours after the injection of spores. In addition to titrating the blood of these animals, the bloods of other guinea pigs that had not been inoculated with spores but that had received antitoxin alone were also titrated. Results are summarized in Table III. Each result recorded in the table represents units of antitoxin on a pool of equal amounts of sera from three guinea pigs.

TABLE III

C. novyi ANTITOXIN TITERS FOLLOWING INTRAPERITONEAL INJECTION OF ANTITOXIN

Days after injection	Spores injected five hours previous to antitoxin		No spores injected	
	500 units in one dose	200 units* + 50 units × 6	500 units in one dose	200 units* + 50 units × 6
1	8 μ/ml.	2.6 μ/ml.	8 μ/ml.	2.6 μ/ml.
4	2.6 "	0.9 "	2.6 "	2.6 "
6	0.9 "	0.7 "	2.0 "	2.0 "
8	0.7 "	0.9 "	0.7 "	2.0 "
10	<0.15 "	0.7 "	0.2 "	0.9 "
13	<0.15 "	0.3 "	<0.15 "	0 9 "
15	<0.15 "	0.7 "	<0.15 "	0.7 "

* *Initial dose 200 units, followed by six doses of 50 units each every 48 hr.*

In the guinea pigs that received a single dose of antitoxin there was less than 0.15 units (lower limit of test) from the 10th to the 13th day on, whereas, as one might expect, antitoxin was present in measurable amounts to the 15th day in the guinea pigs that received the same total amount of antitoxin in divided doses.

Note on Immunization with *novyi* Toxoid

When the study of the possible curative action of *novyi* antitoxin was in progress, work was also undertaken on active immunization with *novyi* toxoid. Only preliminary observations were made and these we are reporting here.

C. novyi toxin (broth filtrate) was detoxified, as in the case of diphtheria and tetanus, by the addition of formalin and storing at 34° to 36° C. until detoxification was complete.

Three doses of the detoxified toxin were injected subcutaneously into each of 22 guinea pigs of about 300 gm. weight, with an interval of two weeks between the first and second injections and three weeks between the second and third. Twenty-five days after the third injection the animals were bled, and five days later each was inoculated intramuscularly with 0.5 ml. of 10^4 dilution of spore suspension.

In Table IV there is summarized antitoxin titers of the blood sera and deaths.

Blood was drawn from the three surviving guinea pigs 4 days and 12 days after inoculation and the sera were titrated for *novyi* antitoxin. In addition, the sera from bloods drawn before inoculation were retitrated at closer intervals (Table V).

TABLE IV

C. novyi TOXOID

ANTITOXIN TITERS AND EFFECT OF SPORE INOCULATION

Guinea pig No.	Antitoxin titer before spore inoculation	Record of deaths					
		Days after spore inoculation					
		1	2	3	4	5	6
3508	<0.06 μ/ml.	–	+				
808	"	–	+				
3980	"	–	+				
3595	"	–	+				
3663	"	–	–	+			
3735	"	–	–	+			
3978	"	–	–	+			
3478	>0.06 <0.3	–	–	+			
3744	"	–	–	+			
509	"	–	–	+			
546	"	–	–	+			
3820	"	–	–	–	+		
158	"	–	–	–	+		
355	"	–	–	–	+		
487	"	–	–	–	+		
3486	>0.3 <1.5	–	+				
3964	"	–	–	–	–	+	
3440	"	–	–	–	–	+	
3519	"	–	–	–	–	–	+
552	"	–	–	–	–	–	–
830	"	–	–	–	–	–	–
998	"	–	–	–	–	–	–

TABLE V

EFFECT OF SPORE INOCULATION ON ANTITOXIN TITER

Guinea pig No.	Antitoxin in blood serum		
	Before inoculation	Four days after inoculation	Twelve days after inoculation
552	1.2 μ/ml.	0.6 μ/ml.	>1.2 μ/ml.
830	0.6 "	0.3 "	>1.2 "
998	0.6 "	0.4 "	>1.2 "

Results in Table V show that the inoculation with spores caused a preliminary drop in antitoxin followed by a rise.

Discussion

The reason for more effective treatment with repeated doses of antitoxin as compared with treatment with the same total amount of antitoxin in a single dose, is no doubt, that the former method ensures effective concentrations of antitoxin (in the order of 1μ per ml.) in the infected animals for a longer period than does the latter.

If one wishes to compare results recorded here with the results of others, it is of course necessary to bear in mind that besides possible differences in strain of *C. novyi* used, there were differences in experimental method. Gordon and McLeod infected guinea pigs by injecting a fresh culture of *C. novyi* into a crushed muscle and treating the animals by administering antitoxin (probably intramuscularly) near the site of infection. The treatment was not successful. Gordon and McLeod were, however, able to prevent infection with *C. novyi* in guinea pigs by administering antitoxin before inoculation. Evans infected guinea pigs by injecting washed organisms from a fresh broth culture into muscle that had previously been injected with a solution of calcium chloride. Antitoxin was administered intravenously and in the early stages of the disease treatment was successful using a single relatively large dose of antitoxin.

Our results are in accord with those of the authors just cited, namely, that antitoxin is effective in controlling experimental infection with *C. novyi*, at least as a preventive agent.

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STUDIES ON THE EFFECT OF SOME AROMATIC AMIDINES AND OTHER CHEMICAL COMPOUNDS ON THE GROWTH OF INFLUENZA VIRUS IN THE EMBRYONATED EGG[1]

By Laurella McClelland and C. E. van Rooyen

Abstract

The effect of chemicals and biological substances on the growth of influenza virus in the embryonated egg is of interest as a fundamental study and as a means of screening substances for their possible chemotherapeutic activities. This work was done to investigate the activity of some heretofore unreported substances. Most chemicals proved to have no effect on the growth of influenza virus in the developing egg, but one group, the aromatic amidines, and hexamidine in particular, was found to possess some inhibitory action. It was an *in vivo* rather than an *in vitro* effect; it was dependent on the route of inoculation and the amount of drug administered; and the activity was exhibited when the introduction of the drug was delayed for as long as 16 hr. after inoculation of the eggs with influenza virus. Preliminary tests have failed to demonstrate any protection of mice infected with influenza and treated with hexamidine.

The inhibitory action of organic and inorganic chemical substances on the growth of viruses in chicken embryos has attracted much attention. Many compounds have been tested for evidence of possible activity against influenza and other viruses in embryonated eggs and experimental animals, but relatively few have been noted to possess antiviral effect (2, 9, 3). Among the few substances that have been reported to show inhibitory effects are nitroakridin (5); chloromycetin (13); apple pectins and other polysaccharides (6). Certain bacterial extracts have also been shown to be active according to Horsfall and McCarty (8).

During the course of our experiments on influenza virus, we have examined a selection of chemical compounds for their inhibitory action, but the bulk of these have proved to be inactive. The present report includes all results and describes in detail some findings obtained with one member of the aromatic amidine series, namely hexamidine, 4 : 4′-diamidinodiphenoxyhexane di-isethionate.

The aromatic amidines have been extensively studied by other workers in connection with the treatment of trypanosomiasis and leishmaniasis. Past investigations have covered a variety of aspects such as the effect of these drugs on human patients, laboratory animals, *in vitro* test tube experiments, and inquiries into their complex pharmacological and metabolic behavior (11, 1, 7, 4, 15, 10, 14).

Materials and Methods

Fertile eggs previously incubated for 11 days were employed throughout in the experiments. The seed virus used was from a stock of frozen allantoic fluid from eggs infected with influenza virus type A, PR8 strain.

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[1] *Manuscript received January 26, 1949.*
Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont. This work was aided by a grant from the National Research Council of Canada.

First, the toxicity of the various drugs to embryos was ascertained. Freshly prepared solutions of the drug in a suitable solvent were inoculated in eggs via the chorioallantoic and yolk sac routes. This procedure was carried out in order to test drugs at an optimum dosage level. To demonstrate the method used, the experimental data on one drug are given in detail as follows. It was found in the case of hexamidine that it was relatively toxic to embryos. When 2 mgm. was injected into the allantoic cavity all the embryos died. When the amount of drug was decreased the number of surviving embryos was increased. In preliminary experiments on small groups of eggs, the average percentage mortality at 1.0 mgm. was 90%; at 0.5 mgm., 70%; at 0.25 mgm., 10%; and at 0.125 mgm., 7%. A test dose of 0.25 mgm. was selected as an amount that did not kill a proportionately high number of embryos as compared to the number that usually die as the result of any injection procedures. Similarly, hexamidine was found to be toxic when administered via the yolk sac route. In this instance, however, a greater amount of drug was required to produce the same percentage mortality and a dose of 0.5 mgm. was selected as the test dose.

Next, influenza virus was injected into the allantoic cavity and this was followed by the administration of the selected dose of drug, either into the same cavity or into the yolk sac. Inoculated eggs were incubated at 36° C. for 44 to 46 hr.; the allantoic fluid was aspirated and tested for evidence of influenza virus by examining the fluid of each egg for its ability to agglutinate fowl red blood cells; thereafter, aliquot volumes of positive fluids were pooled and the haemagglutinin titer of the combined fluids was determined using a modification of the method of Miller and Stanley (12). Hereafter this determination is referred to as C.C.A. (chicken cell agglutination) titer.

Preliminary screening tests showed that there was no evidence of any effect on virus growth when most of the drugs were tested in the manner described above. However, with some of the amidines, and with hexamidine in particular, there was some effect demonstrated when the drug was introduced into the allantoic cavity. Since it may be of interest and assistance to other workers in this field, we have recorded in Table I a list of selected chemicals tried. The data include the tolerated dose of each drug with the route of inoculation and the effect on virus growth. The remainder of the paper deals with the experimental work that was carried out in an effort to evaluate the antagonistic effect shown by hexamidine.

Experiment 1

This was designed to determine whether the action of the drug was an *in vivo* effect upon the growth of influenza virus in the embryo or whether it was due to interference with haemagglutination *in vitro*. Our results showed that hexamidine, in amounts equivalent to those that might be found in the allantoic fluid of drug treated eggs in the present work, when added to the mixture of PR8 infected allantoic fluid and fowl red blood cells did not alter the end point of the titration.

We considered also the possibility of the formation of a metabolic product in drug treated eggs that might have interfered with haemagglutination and so accounted for the fall in haemagglutinin titer. Normal embryonated eggs were injected with 0.25 mgm. of drug and after 48 hr. incubation the allantoic fluid was pooled and tested for the presence of agglutinin-inhibiting substances, but none were found. The control titrations were made using normal allantoic fluid.

TABLE I

Chemical tested	Dose in mgm. and route of inoculation	Result
Benzpyrene	1 (A)* & (YS)†	Negative
Methyl benzanthracene	0.25 (A) & (YS)	Negative
Ethyl benzanthracene	0.25 (A) & (YS)	Negative
Propyl benzanthracene	0.25 (A) & (YS)	Negative
Butyl benzanthracene	0.25 (A) & (YS)	Negative
Amyl benzanthracene	0.25 (A) & (YS)	Negative
Hexyl benzanthracene	0.25 (A) & (YS)	Negative
Dibenzanthracene	0.5 (A) & (YS)	Negative
Thyroxin	0.0003 (A)	Negative
Thiouracil	1.0 (A)	Negative
Vitamin B6	2.0 (A) & (YS)	Negative
p-Aminobenzoic acid	2.0 (A) & (YS)	Negative
Stilboestrol	1.0 (A) & (YS)	Negative
Hexoestrol	0.5 (A) & (YS)	Negative
Propylene glycol	0.02 (A)	Negative
Potassium cyanide	0.25 (A) & (YS)	Negative
Copper sulphate	0.5 (A) and 0.1 (YS)	Negative
Silver nitrate	5.0 (A) & (YS)	Negative
Sodium fluoride	2.0 (A) & (YS)	Negative
Sodium arsenite	0.2 (A) and 0.1 (YS)	Negative
Phenamidine	2.0 (A)	Slight
Stilbamidine	10.0 (A)	Slight
Hydroxystilbamidine	10.0 (A)	Slight
Propamidine	2.0 (A)	Negative
Dibrompropamidine	0.75 (A)	Negative
Pentamidine	0.3 (A)	Active
Hexamidine	0.5 (A)	Active
Heptamidine	0.2 (A)	Active
Octamidine	0.1 (A)	Slight

* *(A) Allantoic.*
† *(YS) Yolk sac.*

The above experiments showed that the low haemagglutinin titer found in hexamidine treated eggs was not due to the direct action of hexamidine on red cell agglutination or to the presence of antagonistic substances in the allantoic fluid.

Experiment 2

This was designed to find whether hexamidine exhibited any direct *in vitro* antiviral activity.

To a 10^{-3} dilution of PR8 virus in Ringer's solution, enough drug was added to make a final concentration of 0.25 mgm. per ml. The mixture was incubated at 37° C. for two hours and from it were made five 10-fold dilutions up to 10^{-8}. Eggs were inoculated with each dilution to determine the 50% infective dose (ID_{50}) and to compare the results of red cell agglutination titers with eggs that had received an identical range of virus dilutions prepared as above but without hexamidine. The results (Table II) indicated that although the drug was not comparable in effect to that of an antiseptic such as formalin, there was some *in vitro* effect as evidenced by the shift in the ID_{50}.

TABLE II

In vitro EFFECT OF 0.25 MGM. HEXAMIDINE ON INFLUENZA VIRUS AS DEMONSTRATED BY INFLUENCE ON INFECTIVITY TO EMBRYONATED EGGS AND C.C.A. TITER

Virus dilution*	First test				Second test			
	Ratio of positive eggs to total eggs tested		C.C.A. titer of positive eggs		Ratio of positive eggs to total eggs tested		C.C A. titer of positive eggs	
	Drug treated inoculum	Control inoculum	Drug treated inoculum	Control inoculum	Drug treated inoculum	Control inoculum	Drug treated inoculum	Control inoculum
10^{-4}	6/10	8/10	1/676	1/708	10/10	9/10	1/407	1/432
10^{-5}	4/9	9/10	1/624	1/1024	9/9	9/9	1/394	1/468
10^{-6}	3/9	7/9	1/1024	1/1024	7/9	9/10	1/380	1/537
10^{-7}	0/10	4/9	—	1/841	2/8	4/8	1/403	1/474
10^{-8}	0/10	0/9	—	—	0/9	1/10	—	1/339

* *Dose of virus dilution was 0.2 ml. per egg.*

ID_{50} of drug treated inoculum as compared to control inoculum in first test, $10^{-4.8}$ to $10^{-6.4}$; in second test, $10^{-6.5}$ to 10^{-7}.

Experiment 3

Table III shows the effect of the administration of diminishing doses of drug ranging from 1 mgm. to 0.015 mgm. to eggs previously inoculated with a fixed amount of virus (0.2 ml. of a 10^{-6} dilution that by previous titrations was shown to be between 1 and 10 ID_{50}). The results would indicate that a direct correlation existed between the amount of drug introduced and the resulting haemagglutinin titer of the allantoic fluid.

Experiment 4

In previous tests it was shown that the drug was able to inhibit the growth of influenza virus when eggs were inoculated with 1 to 10 ID_{50} doses of virus. In the present test, it was shown that the drug was also potent against still higher doses of virus. Table IV presents the results. It should be noted

TABLE III

Effects of decreasing amounts of hexamidine on the presence of virus in the allantoic fluid and on the C.C.A. titer of pooled positive fluids: two different experiments are recorded

Test No.	Dose of drug in mgm.	No. of eggs inoculated	Ratio of positive eggs to total eggs tested	C.C.A. titer of pooled positive fluids
A	1.0	10	0/3[*]	—
	0.5	10	1/5	1/44
	0.25	9	7/9	1/93
	0.125	10	7/8	1/299
	0.06	10	8/9	1/417
	0.03	10	9/9	1/367
	0.015	9	8/9	1/549
	0 (Control)	9	7/8	1/512
B	0.25	12	11/12	1/128
	0.125	12	12/12	1/221
	0.06	12	12/12	1/309
	0.03	11	11/11	1/363
	0 (Control)	11	9/11	1/550

[*] *The toxicity of the drug can be gauged by comparing the number of surviving eggs tested with the number of eggs inoculated.*

TABLE IV

Effect of 0.25 mgm. hexamidine on increasing doses of virus, tested *in vivo*

Dose of inoculum in ID_{50}'s	Ratio of positive eggs to total eggs tested		C.C.A. titer of pooled positive fluids	
	Drug treated	Control	Drug treated	Control
1	0/9	3/10	—	1/256
10	3/6	9/10	1/38	1/367
100	8/9	8/9	1/73	1/335
1000	9/9	9/9	1/93	1/320
10,000	8/9	9/9	1/86	1/335

that the action of the drug was most pronounced on small dosage of virus. Thus, when small amounts of virus were administered to the egg, both the number of eggs infected and the corresponding C.C.A. titer were markedly reduced. On the other hand, when large doses of virus were inoculated, the effect was reflected only in the diminished C.C.A. titers of positive fluids.

Experiment 5

This series was planned to explore the effect of administering hexamidine at time intervals before and after infection with virus.

Table V contains a summary of findings. The results show that the drug is active if administered either two and one-half hours before introduction of virus or as late as 16 hr. after virus inoculation.

TABLE V

EFFECT OF ADMINISTERING HEXAMIDINE BEFORE AND AFTER INOCULATION OF VIRUS

Test No.	Interval between injection of drug and inoculation of virus*	Number of eggs inoculated	Ratio of positive eggs to total eggs tested	C.C.A. titer of pooled positive fluids
1	Drug 2½ hr. before virus	19	17/18	1/64
	Drug within 5 min.	19	15/18	1/82
	Drug 5 hr. after virus	20	15/15	1/59
	Control	19	17/18	1/473
2	Drug 2½ hr. before virus	16	13/15	1/116
	Drug within 5 min.	16	14/14	1/94
	Drug 5 hr. after virus	16	12/13	1/170
	Control (for above)	16	12/15	1/512
	Drug 16 hr. after virus	16	13/14	1/146
	Control (for 16 hr. test)	16	13/16	1/512

* *Dose of drug: 0.25 mgm. per egg; dose of virus: 0.2 ml. of* 10^{-6} *dilution of virus. Both inoculations made into allantoic cavity.*

Experiment 6

This experiment was an attempt to determine the influence of hexamidine on the normal growth curve of influenza virus in the embryonated egg. Eggs treated with 0.25 mgm. of hexamidine and untreated control eggs were inoculated with 0.2 ml. of a 10^{-6} virus dilution (1 to 10 ID_{50}) and were incubated. Throughout the next 48 hr., samples of allantoic fluid were aspirated at four hourly intervals and were tested for presence of virus. Control eggs first showed red cell agglutination after 28 hr. incubation, but in drug treated eggs, such response was delayed for as long as 40 hr. In order to ascertain the extent of delay in the multiplication of virus, a second experiment was performed. Three groups of drug treated eggs were inoculated with influenza virus and incubated for 30, 48, and 72 hr. respectively. Thereafter, the eggs were removed from the incubator, chilled by being placed in ice cold water, and the allantoic fluid was aspirated. This was tested for the presence of virus and the C.C.A. titer was measured. Appropriate controls were incorporated. Findings are summarized in Table VI. It should be noted that notwithstanding the prolonged incubation of eggs for an additional 24 hr. more than the usual 48 hr., the C.C.A. titer of the drug treated eggs remained low as compared to the titer of the control eggs.

Experiment 7

It is recognized that the infectivity of allantoic fluid containing viable virus is roughly proportional to the chicken cell agglutination titer of that fluid, but it is also known that fluids that do not produce agglutination of red blood cells may be infective. Therefore, an embryo titration enables one to estimate more precisely the amount of viable virus present in any preparation. To determine the extent of the diminution of the amount of virus in fluids from drug treated eggs, the fluids were titrated in eggs and the 50% end points

TABLE VI

EFFECT OF PROLONGED INCUBATION ON VIRUS CONTENT IN ALLANTOIC FLUID OF HEXAMIDINE TREATED AND UNTREATED EGGS

Hours of incubation	Ratio of positive eggs to total eggs tested		C.C.A. titer of pooled positive fluids	
	Treated*	Control†	Treated	Control
30	0/11	6/13	—	1/226
48	7/12	10/13	1/61	1/462
72	12/12	9/12	1/54	1/407

* *Treated eggs received 0.25 mgm. hexamidine and 0.2 ml. of a* 10^{-6} *dilution PR8 influenza simultaneously.*

† *Control eggs received 0.2 ml. of a* 10^{-6} *dilution PR8 influenza.*

were estimated. The percentage loss in infectivity was then calculated, taking as 100% the end point titer of the fluids of infected, untreated eggs. Results of such titrations and the calculations of virus loss are presented in Table VII.

TABLE VII

COMPARISON OF C.C.A. TITER WITH ID_{50} OF ALLANTOIC FLUIDS OF UNTREATED (CONTROL) AND DRUG TREATED EGGS

Untreated or treated	C.C.A. titer of pooled fluids	ID_{50} of pooled fluids	Percentage virus present	Percentage diminution of virus
U*	1/316	9.280	100	
T†	> 1/2	6.822	0.35	99.65
U	1/330	9.78	100	
T	> 1/2	8.48	5.01	95
U	1/330	9.44	100	
T	> 1/2	8.5	10.14	89.86
U	1/330	9.63	100	
T	> 1/2	7.75	1.318	98.682
U	1/468	9.50	100	
T	> 1/2	7.423	0.83	99.17

* *Untreated.*

† *Treated.*

Discussion

Although many of the chemicals tested were ineffective against influenza virus, the most active drug encountered was 4 : 4′-diamidinodiphenoxyhexane diisethionate (hexamidine), a member of the aromatic amidine group of compounds, the pharmacological action of which in man and animals has already been studied.

Our tests have shown that the toxicity of the drug to chicken embryos varied according to the route of administration, being more toxic by the allantoic route than by the yolk sac. Likewise, the activity of the drug against influenza virus depended on the route of inoculation, being active by the allantoic route only.

Inquiry into the time factor showed that the drug was able to inhibit the development of virus when it was administered as long as 16 hr. after introduction of virus. Further work along these lines may yield information concerning the mechanism by which hexamidine interferes with the multiplication of influenza virus in the embryonated egg.

Experiments indicated that the neutralizing effect of hexamidine on influenza virus was best shown when an adequate amount of drug was administered in the presence of a small dosage of virus, although some action was also evident with larger virus and smaller drug dosage. This effect is exhibited by most chemotherapeutic agents, i.e., an adequate drug dosage must be administered to cope with the infecting agent, and it is important to remember this when chemotherapeutic screening tests are performed with eggs.

The loss of infectivity of low C.C.A. titer fluids from eggs that had been treated presents a more quantitative measure of virus inhibition. Although one was disappointed not to find complete absence of virus in fluids that showed no chicken cell agglutination, there was found to be a loss of from 99.65 to 89.86% of virus in such fluids. In using chicken cell agglutination in screening tests it would appear advisable to employ it as an indicator only, and, as the final criterion of inhibition of virus growth, to rely on the more sensitive test, that of the infectivity of the fluid in question.

Preliminary experiments on the chemotherapeutic effect of hexamidine when administered subcutaneously to mice have failed to demonstrate any protection against infection with influenza. Although this is disappointing it should be remembered that the mechanism of virus multiplication is not known and that although a compound may inhibit growth of a virus in chick embryo protein, it does not necessarily follow that the same response should be expected from adult mouse tissue.

Acknowledgments

The authors are indebted to Dr. A. J. Ewins, of Messrs. May and Baker, London, for a generous supply of amidines, also to Dr. Alexander Haddow, of the Royal Cancer Hospital, London, for carcinogenic compounds. We owe our thanks to Miss E. K. Campbell and Miss Greta Nicolson for their technical assistance, and to Dr. Angus F. Graham for much helpful criticism.

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AN ELECTRIC CAUTER AS A STERILITY AID IN INOCULATION OF EGGS[1]

By N. A. Labzoffsky

Abstract

An electric cauter for sterilization of the opening through which embryonated egg is inoculated is described. The apparatus consists of a transformer, a rheostat, two leads, and a nichrome wire tip; the latter serves as a hot point. The temperature that develops at the tip is approximately 1000° C. and, therefore, instantaneous application of the hot point to the hole in the egg shell results in absolute sterilization of the area.

It is generally known among laboratory workers that application of iodine or any other disinfectant to the shell, to sterilize the opening through which the embryonated egg is inoculated, does not ensure the desired sterility at the site of inoculation and may result in a significant percentage of contaminations when a large number of eggs is being inoculated.

To overcome this, a simple and highly efficient electric cauter has been employed in our laboratory, which reduces contamination of the eggs during inoculation to practically nil. The cauter, of course, is suitable only for the air sac route inoculation.

The apparatus consists of a transformer (110 v. primary, 6 amp., 2.5 secondary), a rheostat (100 ohm, 25 to 50 w.), two leads, and a tip. The construction of the apparatus can be followed in detail by reference to Fig. 1.

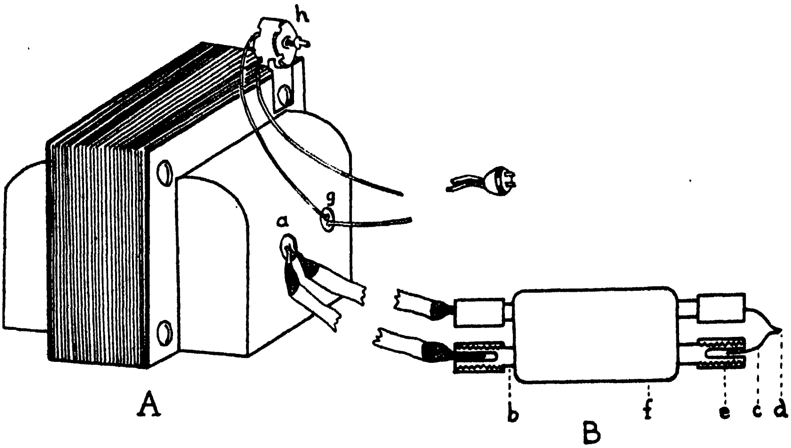

FIG. 1. *A* = *Transformer.* *B* = *Cauter.*

Each lead, preferably flexible braided electrical cable of low resistance (known as "tinned copper shielding"), is approximately 3 ft. long and is

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[1] *Manuscript received March 4, 1949.*

Contribution from the Virus Section, Division of Laboratories, Ontario Department of Public Health, Toronto, Ont.

incased in a rubber tubing of a suitable diameter for insulation. One end of each lead is connected to the secondary winding of the transformer (Fig. 1 A, *a*) and the other end to a brass rod (B, *b*). The brass rods are approximately 3/16 in. in diameter and 3 in. long. The free ends of the brass rods are connected to each other by nichrome wire (*c*), which serves as a hot point. The nichrome wire, gauge 22 B & S, is about 1 in. long and bent in the middle at an acute angle to form a sharp point (*d*). To facilitate the assembly of the apparatus, as well as to provide a proper contact, the ends of the rods are threaded and slotted to a depth of ½ in. Screw type sleeves (*e*) go over the ends of the rods so that when the end of the lead or the nichrome wire is inserted into the slot, a low resistance connection is obtained by tightly screwing the sleeve. The rods are ¼ in. or so apart and are embedded in plaster of Paris (*f*).

The primary winding of the transformer (*g*) is connected to an electric cord with a plug and rheostat (*h*), which is mounted on the transformer.

For operation, the apparatus is plugged into an electric outlet and the temperature of the point is adjusted to the first visible white heat with the rheostat. The eggs are drilled in the usual manner with some sharp instrument without breaking the shell membrane, and the opening is cauterized by touching it with the hot point. The temperature that develops at the point is approximately 1000° C. and therefore instantaneous application of the point to the opening ensures absolute sterilization of the area.

By using tungsten wire of a suitable gauge, instead of nichrome wire, it may be possible to obtain a sufficiently high temperature at the point to burn holes through the shell without preliminary drilling.

An electrically heated wax pencil for sealing inoculated eggs, which we found to be a very convenient and time-saving device, has been described elsewhere (1).

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A SIMPLE GAS ANALYZER[1]

By F. E. J. Fry

WITH AN APPENDIX

By A. C. Burton and O. G. Edholm

Abstract

The gas analysis apparatus described is similar to an enlarged version of Krogh's microapparatus. It consists of a 1 cc. burette calibrated in one-hundredths, enclosed in a water jacket joined above to a glass funnel through a short capillary and below to a mercury reservoir. The absorbing solutions are placed in the glass funnel above, and the gas sample is injected through a needle inserted into a rubber cap on a side arm of the mercury reservoir.

The apparatus is simple in design and in use, is easy to clean, and the cost is low. Accuracy is approximately ± 0.5%. A modified analyzer has been used by students with satisfactory results.

Introduction

Analyzers of the type described here were first constructed early in 1942 and used extensively by investigators of the Royal Canadian Air Force in the study of oxygen apparatus. These analyses were routinely carried out in decompression chambers at altitudes up to 40,000 ft. and occasionally in aircraft as well as under more conventional laboratory conditions. The apparatus proved extremely convenient and reliable and its operation is so simple that an inexperienced person can produce fair results after one or two trials. An analysis requires about five minutes and if care is taken to allow for complete drainage, the probable error of a single determination is about 0.05% when the gas measured is 20% of the total volume of the sample.

Construction

Working drawings for the construction of the analyzer are given in Fig. 1. It consists of a calibrated glass tube of about 3 mm. bore and 25 cm. long, with a capillary of about 1 mm. bore and 5 cm. long attached to one end and a bulb of a little more than 5 cc. capacity blown on the other. A cup of about 2 to 3 cc. capacity is blown on the free end of the capillary. Two short arms are fused to the 5 cc. bulb, one of these being opposite the calibrated tube and the other near the first arm and making an angle of about 45° with it. The arm in line with the capillary is about 12 mm. in diameter and 3 cm. long, the side arm has a diameter of about 6 mm. and is less than 1 cm. long. Both are flared at the open end.

The zero mark for the calibrated section is arranged to come about one-third of the way up the 1 mm. capillary. The total volume of the calibrated

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[1] *Manuscript received December 13, 1948.*

Contribution from the Department of Zoology, University of Toronto, Toronto, Ont. and the Department of Physiology, University of Western Ontario, London, Ont.

portion is 1 cc. and the graduations are one-hundredths. This portion can be conveniently made from a 1 cc. pipette cut off at the 0.01 cc. mark for attachment to the capillary. It is then necessary only to determine the position of the zero mark on the capillary to complete the calibration.

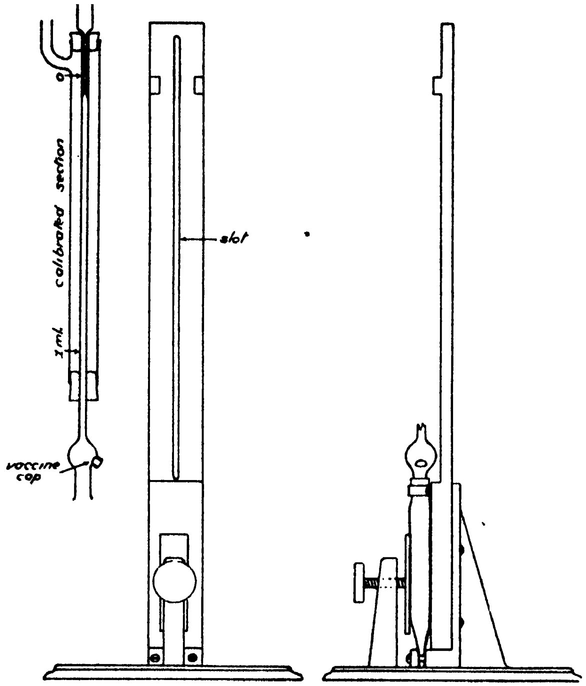

FIG. 1. *Drawing of apparatus. The glass parts are shown on the left. The side arm on the water jacket can be replaced by making an opening in the upper cork. The stand and reservoir are shown in two aspects. The base is of metal, the upright is wooden.*

The calibrated tube is provided with a water jacket. It is convenient to slip a rubber stopper in place around the pipette before attaching the bulb to close the lower end of the water jacket. A split stopper can be used at the upper end. If desired, the jacket can be blown integrally with the pipette.

A piece of medium wall rubber tubing about 12 cm. long is bound tightly to the terminal arm on the bulb and the apparatus is mounted vertically on a suitable board with the cup uppermost. Synthetic rubber tubing should not be used. The free end of the rubber tube is closed by a clamp on the base. A suitable screw adjustment is provided for flattening the tube with a metal tongue.

The side arm of the bulb is provided with a rubber cap soft enough to allow a No. 26 or 27 hypodermic needle to penetrate it and thick enough to prevent more than a droplet of mercury from forcing its way through the puncture on the withdrawal of the needle. A vaccine cap is convenient for this purpose.

Operation

Directions for operation, designed as a laboratory sheet for students, are given in the appendix.

The solutions used consist of $N/10$ potassium hydroxide for the absorption of carbon dioxide and sodium anthraquinone-β-sulphonate for the absorption of oxygen. This last solution is prepared from a mixture of one part sodium anthraquinone-β-sulphonate to five parts of sodium hydrosulphite dissolved in $N/10$ potassium hydroxide. The solution should be prepared fresh at least every day.

Latterly, indigo carmine has been substituted for the anthraquinone, using the same concentration. It has the advantage of being more stable.

Acknowledgments

The original apparatus was constructed in No. 1 Clinical Investigation Unit of the Royal Canadian Air Force, the funds being made available through the Associate Committee of Aviation Medicine of the National Research Council of Canada. The apparatus, as no doubt will be recognized, is more or less an enlarged version of Krogh's (1) microapparatus. The hypodermic method of introducing the sample was learned from Dr. P. F. Scholander of Swarthmore College.

Reference

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APPENDIX

Use of the Fry Analyzer for Student Classes

The apparatus designed by Dr. Fry has been used in the practical physiology class for medical students with satisfactory results.

One advantage of this apparatus is the low cost. The water jacket was constructed without internal seals or side arm, being closed by rubber corks above and below. The upper cork has a split in it for filling the jacket and to allow for changes in volume.

TABLE I

Cost of student apparatus

Total cost for:	
24 Analyzers	$212.50
Each	8.85
Detailed cost for 24 analyzers:	
Metal parts	24.80
Glass parts	31.00
Wood	10.00
Labor	
Wood work	31.00
Glass blowing	60.00
Assembly	55.70

Twenty-five sets were constructed at a cost of $8.85 for each analyzer (excluding mercury). The work was carried out in the University workshops and includes full charge for labor. Table I gives the details of the expenses.

The results of gas analyses by students at their first trial are shown in Fig. 2.

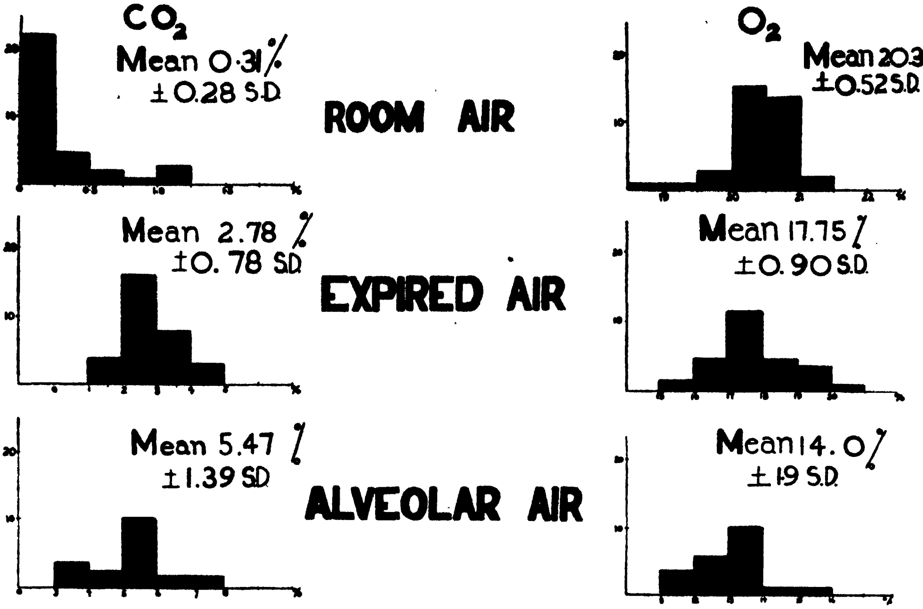


FIG. 2. *Results obtained by students at first trial. All students analyzed the same room air, and also the same expired air, which was collected in a Douglas Bag. A check analysis showed the composition was: carbon dioxide 2.9%; oxygen 17.6%. The alveolar air samples were collected individually using a Haldane tube. The material analyzed might therefore differ considerably. Error in oxygen determination is greater than carbon dioxide determination as two operations are involved.*

It should be emphasized that these results were obtained after a brief demonstration and were the first gas analyses performed by the students. Reproduced below is the instruction sheet issued to the class. Fig. 3 shows the diagrams issued to illustrate the technique.

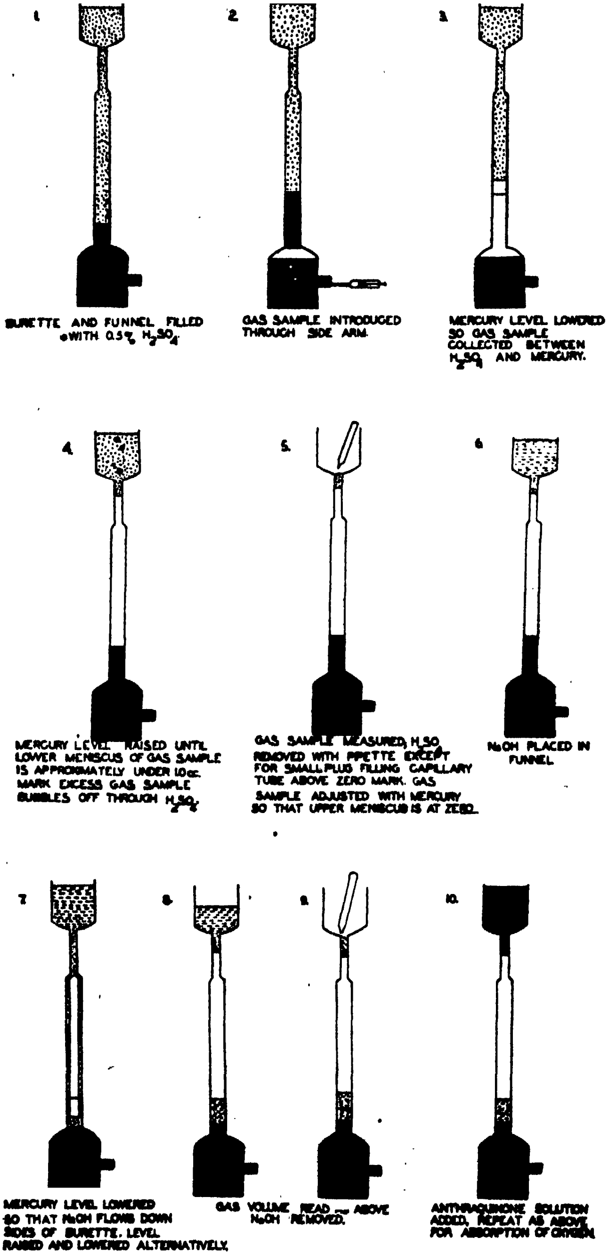
The advantages of this apparatus for student use are simplicity, absence of taps, ease of cleaning, and low cost. The main source of error in student hands is the risk of losing part of the gas sample by too sudden movements of the screw controlling the mercury level.

The use of the analyzer in classwork means that the student does not have to spend an undue amount of time in learning technique and can obtain valid results in experiments on respiration with comparative ease.

Instruction Sheet

THE ANALYSIS OF RESPIRATORY GASES

The apparatus used is known as the Fry Analyzer, after the name of the inventor. It is one of many types of apparatus, but has the advantage of relative simplicity. The principle of this, as with other types, is that a sample of gas is introduced and its volume (*A*) accurately measured. The carbon dioxide is absorbed by bringing the gas into contact with sodium hydroxide, and the volume (*B*) of gas left is measured—the difference of $A - B$ = volume of carbon dioxide. Oxygen is then absorbed with



1. BURETTE AND FUNNEL FILLED WITH 0.5% H_2SO_4.

2. GAS SAMPLE INTRODUCED THROUGH SIDE ARM.

3. MERCURY LEVEL LOWERED SO GAS SAMPLE COLLECTED BETWEEN H_2SO_4 AND MERCURY.

4. MERCURY LEVEL RAISED UNTIL LOWER MENISCUS OF GAS SAMPLE IS APPROXIMATELY UNDER 10cc. MARK EXCESS GAS SAMPLE BUBBLES OFF THROUGH H_2SO_4.

5. GAS SAMPLE MEASURED, H_2SO_4 REMOVED WITH PIPETTE EXCEPT FOR SMALL PLUG FILLING CAPILLARY TUBE ABOVE ZERO MARK. GAS SAMPLE ADJUSTED WITH MERCURY SO THAT UPPER MENISCUS IS AT ZERO.

6. NaOH PLACED IN FUNNEL

7. MERCURY LEVEL LOWERED SO THAT NaOH FLOWS DOWN SIDES OF BURETTE. LEVEL RAISED AND LOWERED ALTERNATIVELY.

8. GAS VOLUME READ — ABOVE

9. NaOH REMOVED.

10. ANTHRAQUINONE SOLUTION ADDED, REPEAT AS ABOVE FOR ABSORPTION OF OXYGEN.

FIG. 3. *Diagrams illustrating the various stages in the analysis.*

anthraquinone and the final volume (*C*) measured, $B - C$ = volume of oxygen. The remainder is assumed to consist of nitrogen. The rare gases are not considered.

DIRECTIONS

1. Raise mercury level by turning screw at base until it reaches junction of burette and funnel.

2. Pipette acid water (0.5% sulphuric acid) into the funnel at the top of the apparatus.

3. Lower mercury level by means of screw so that funnel and burette are filled with acid, and mercury stands 1 cm. approximately above the bulb of the burette.

4. Introduction of gas. The first analysis will be of room air. A hypodermic syringe is filled with air by repeated filling and emptying so that a representative sample is obtained. From this point on, the plunger must not be withdrawn. A needle is put on the syringe and enough gas expressed to wash out bore of needle. The needle is pushed through the small rubber cap on the side of the burette bulb and 2 to 3 cc. of gas introduced. The mercury will rise in the burette trapping the gas in the bulb.

5. Lower the mercury in the burette by means of the screw until it has all come back into the reservoir.

6. Measurement of sample. Raise the gas sample into the burette by means of the screw until the water meniscus is approximately at the 0.95 cc. mark. Excess gas will escape through the funnel. Remove acid water in funnel with pipette so that level of acid water is at junction of burette and funnel. *Caution:* Care must be taken that none of the gas sample escapes during this procedure (i.e. do not place pipette so that it sucks directly from capillary beneath funnel).

Adjust the mercury level so that the upper level of the gas sample is brought to the zero mark (i.e. the scratch mark between calibration and funnel). Read the level of the lower meniscus estimating to nearest ½ small division (1/200 cc.). This is Volume *A*.

7. Pipette about 1 cc. *N*/10 potassium hydroxide solution into the reservoir. *Note:* Use pipettes for only one solution (i.e. do not use the pipette with which the sulphuric acid was put into the reservoir for the potassium hydroxide).

8. Mix the potassium hydroxide with the gas sample by alternately lowering and raising the mercury level. The potassium hydroxide will run down the sides of the burette. Finally, lower the mercury so that the gas sample is brought down to the bulb. Measure the gas sample as above without removing excess potassium hydroxide. Mix again and remeasure to see that there has been no further absorption. If the two readings are identical, remove excess potassium hydroxide from funnel and make final reading of Volume (*B*).

Then $A - B$ = volume of carbon dioxide and $\frac{A - B}{A} \times 100$ = percentage of carbon dioxide.

9. Pipette anthraquinone solution into funnel and carry out the same procedure as in 8, for the absorption of oxygen. *Caution*: Do not leave anthraquinone solution unstoppered. Final volume is (C).

Then $B - C$ = volume of oxygen and $\frac{B - C}{A} \times 100$ = percentage of oxygen.

10. The final volume after absorption is (C). C = volume of nitrogen.

Then $\frac{C}{A} \times 100$ = percentage of nitrogen.

11. At the end of the analysis the apparatus is washed twice with 0.5% sulphuric acid. The pipette is filled with acid and is ready for the next analysis.

Precautions

1. Final measurements must always be done under the same conditions.
2. Do not warm the instrument with your hand. This will alter the volume of gas.
3. When readings are made, after mixing samples, wait for one minute to allow fluid within the burette to drain to the bottom.
4. In mixing, care must be taken that none of the gas sample escapes by bubbling through the reservoir.

THE LIPIDOLYTIC ENZYMES OF LARVAE OF *GALLERIA MELLONELLA*[1]

By Edith Mankiewicz

Abstract

The lipidolytic enzymes of *Galleria mellonella* and of pancreas are strongly contrasted by their activity on different substrates. The former is distinguished by its action on compounds of higher alcohols with lower fatty acids. More effective methods of extraction and preservation of the enzymes are described. The influence of emulsifying agents on the activity of the enzymes is demonstrated. The activity of *Galleria mellonella* enzyme on the wax of *Mycobacterium tuberculosis* is a particular feature.

More than three decades ago, Metalnikov discovered the exceptional ability of the digestive system and the serum of larvae of *Galleria mellonella* (L.G.M.) to digest wax and the waxy envelope of the tubercle bacillus. Although this observation suggests various possibilities, relatively few publications followed those of Metalnikov (9, 10, 11, 12, 13, 14).

In 1928, V. Pertzoff (15) extracted the powdered acetone-dried larvae with glycerol and sodium chloride solution, and he observed hydrolysis of olive oil and beeswax, and, at 45° C., a hydrolyzing action on the bacilli of tuberculosis. N. Fiessinger and A. Gajdos (6) tried unsuccessfully to purify the glycerol extract of the ground up L.G.M. by adsorption and elution, using the method of Willstaetter (17, 18). Their glycerol extract acted on tributyrin but did not act on olive oil. It was most active at room temperature and at pH 7.0 to 7.8. These authors pointed out one of the difficulties encountered when working with the glycerol extract of the L.G.M. to be the loss of more than 78% of its activity within the first 10 days after its preparation. C. Manunta and S. Carhoni (8) tested the fats extracted from the isolated intestines of the larvae to show that acidity, the saponification number, and the iodine value were all higher than those of beeswax, which confirms Metalnikov's observation of a digestion of alimentary wax in the intestine of the larvae. The nature of the lipases in the L.G.M. have not yet been well defined, but G. Clement and A. M. Frisch (4) found important quantities of lecithinase, which splits off the fatty acids of lecithin, and a cholesterase, which, at pH 7.2, converts cholesterol into its fatty acid esters.

The fact that the number of investigations of the lipidolytic enzymes of the L.G.M. is so restricted might readily be explained by the technical difficulties that are involved. To appreciate them it is necessary to consider briefly those that complicate the work on lipatic enzymes in general. Besides a quite remarkable confusion in the terminology concerning "esterases", "lipases", "hydrolysis", and "saponification*", many authors neglect to

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[1] *Manuscript received March 5, 1949.*

* *Bull (3) recommended that the term saponification be limited to hydrolysis in which glycerol and soaps are obtained as the ultimate products, and the general name of hydrolysis be applied to all reactions in which fatty acids are split from glyceride with the addition of water.*

define the conditions of their experimental work. For instance, the controversy about the diminution of blood lipases during tuberculosis is due, at least partly, to the fact that different substrates have been used for investigations. According to our own experience in evolutive tuberculosis in guinea pigs, the various blood serum lipases do not diminish in the same proportion. Thus, in advanced cases of tuberculosis, the splitting of benzyl butyrate and ethyl butyrate show a marked decrease (loss of 28%), whereas the blood serum did not lose nearly as much enzymatic activity on olive oil (loss of 2%).

In reality, most of the lipolytic systems that have so far been studied represent a spectrum of activity, the composition of which varies with the conditions of experiment. To allow comparison and classification of the lipidolytic enzymes, we have to consider the following factors: method of preparation and degree of purification; activity on different substrates under various conditions of temperature and pH; resistance to antiseptics and inhibitors; and, as we shall see, the influence of emulsifying agents on activity and preservation of the preparation.

Some special difficulties are encountered in working with L.G.M., the first of which is to obtain a sufficient supply of larvae. An average of 20 to 30 adult larvae, ground and dried according to Willstaetter's method for pancreas powder, are needed to obtain 1 gm. of powder, or 16 cc. of glycerol extract. Through the kindness of Mr. S. M. Deschênes and Mr. Methot of the Department of Agriculture (Ottawa) infected beehives were obtained and the adult larvae collected.

After experimenting with different methods of extraction, including those of Willstaetter and Waldschmidt-Leitz, Fiessinger, Glick, and King, modified according to J. Desbordes (5), the extraction of 1 gm. of the powder by 16 cc. of pure glycerol at room temperature during three days seemed at first the most efficient method (Table I). However, later experiments proved the advantage of adding an emulsifying agent to the glycerol.

TABLE I

EXTRACTS OF THE ACETONE-DRIED POWDER OF L.G.M. IN UNITS PER 100 CC.

Substrate	Extractives		
	NaCl 0.85%	NaCl 10%	Glycerol
Ethyl-*n*-butyrate	100	60	307
Benzyl-*n*-butyrate	95	55	300
Tributyrin	210	155	540
Olive oil	90	70	290
Beeswax	55	20	310
Suspension of Tb bacilli	60	23	292

Note: Carboxel H.V. 1.5% as emulsifying agent during reaction. Initial pH 7.2. Titration after 24 hr. at 37° C.

Like Fiessinger, we failed in the adsorption and elution of the extract by aluminum hydrate.

In their *Studies of pancreatic function* (7), N. P. Goldstein and J. H. Roe discuss numerous methods for determining the activity of lipidolytic enzymes. The titration method using 0.1 *N* alcoholic potassium hydroxide, as proposed by these authors, was adopted as the most convenient, and sodium diethyl barbiturate was used as a buffer. Calcium acetate was added on the supposition that the fatty acids liberated in the hydrolysis form an insoluble calcium salt and that this removal of one of the end products prevents the reversal of the reaction. Using a standard amount, 1 cc. of the filtered enzyme preparation (equal to 1/16 gm. of powder), and allowing 24 hr. at 37° C. before titration, our only modification of the method proposed by Goldstein and Roe was the substitution of their emulsifying agent (bile glycerol) by gum of acacia or "Carboxel" when working with extracts of L.G.M. The results are expressed in arbitrary units determined by the number of cc. of 0.1 *N* potassium hydroxide to produce permanent pink to phenolphthalein end point minus the titration of the blank (containing heat-inactivated enzyme preparation) multiplied by 100: this figure gives the number of esterase or lipase units per 100 cc. of the preparation.

Comparison between the lipidolytic activities of glycerol extracts of beef pancreas and of L.G.M. are shown in Tables II and III.

TABLE II

COMPARISON OF HYDROLYTIC ACTIVITIES OF GLYCEROL EXTRACTS IN UNITS PER 100 CC.

Substrate	Beef pancreas		L.G.M.	
	Emulsifying agent during reaction			
	Glycerol-bile	Gum acacia 20%	Glycerol-bile	Gum acacia 20%
Ethyl-*n*-butyrate	310	150	120	307
Benzyl-*n*-butyrate	910	415	98	297
Tributyrin	1525	1350	150	514
Olive oil	550	515	150	266
Beeswax	0	40	115	300

Note: Initial pH 7.2. Titration after 24 hr. at 37° C.

It is evident from the results obtained that the lipidolytic activities of beef pancreas and L.G.M. do not cover the same substrates. Whereas the pancreas extract is most active on tributyrin, olive oil, and benzylbutyrate, the extract of L.G.M. has its highest activity on tributyrin and beeswax. The action of pancreas on tubercle bacilli is practically nil, and it has only little action on the alcohol-ether extract of the bacilli.

The specific differences in the compared activities of both extracts became more marked with the use of emulsifying agents other than bile glycerol. In

TABLE III

COMPARISON OF HYDROLYTIC ACTIVITIES OF GLYCEROL EXTRACTS IN UNITS PER 100 CC.

Substrate*	Beef pancreas		L.G.M.	
	Emulsifying agent during reaction			
	Glycerol-bile	Gum acacia 20%	Glycerol-bile	Gum acacia 20%
Suspension of tubercle bacilli in saline	10 (?)	0	85	278
Alcohol-ether extract of Tb bacilli ("Phosphatides - low melting wax-acetone soluble fats")	30	40	42	110
Chloroform-ether extract of Tb bacilli ("purified wax")	0	20	35	93
HCl - chloroform - ether extract, freed from HCl ("firmly bound lipids")	0	0	27	56

Note: Initial pH 7.2. Titration after 24 hr. at 37° C.

* *A human strain of tubercle bacilli was grown on the medium of Proskauer and Beck, modified by Youmans and Karlson. Four- to six-weeks-old cultures were filtered, the bacilli were washed and thoroughly emulsified in saline. The fat fractions were prepared according to Anderson (1): the extracts were filtered and reduced. The values we indicate in these series of experiments have only comparative value: beef pancreas/LGM, since further purifications of the fractions might cause a shift in these "units".*

an attempt to intensify the lipidolytic action of the extracts, bile, bile salts, albumen, and gum of acacia were used.

On the kind suggestion of Prof. Labarre (Université de Montréal), we tried "Carboxel" (sodium carboxymethyl cellulose), which is a relatively pure product compared to gum of acacia.

The addition of bile, bile salts, and gum of acacia gave the following results:

(1). Bile solution in glycerol prepared according to Balls, Matlack, and Tucker (2) intensified the action of *pancreas extracts* on benzyl butyrate, and also on tributyrin, ethyl butyrate, and olive oil. Cholic acid was the most activating bile acid for pancreas lipases and esterases. (These findings are similar to those of Balls, Matlack, and Tucker (2), E. Rothlin and W. R. Schalch (16), and others.)

(2). The enzymatic activity of the *extracts of L.G.M.* was not favored by the addition of bile or bile salts, either in regard to their action on neutral fats or when the substrate was beeswax, emulsion of tubercle bacilli, or extracted tubercle bacilli fat fractions.

(3). Gum acacia, 20%, pH adjusted to 7.2, added to the *pancreas extracts* inhibited the cleavage of ethyl butyrate, benzyl butyrate, tributyrin, and slowed down the hydrolysis of olive oil, but allowed a faint action on beeswax, and on one group of lipoids extracted from tubercle bacilli.

TABLE IV

Loss of activity of various extracts of L.G.M. in units per 100 cc.

Residual activity titrated after 24 hr. at 37° C. on various substrates. During the reaction, 1 cc. of each extract was emulsified in gum of acacia 20%. Initial pH 7.0 to 7.2

Days after preparation of extracts	Extractives					
	Glycerol	Glycerol + gum	Glycerol + carboxel H.V. 3%	Glycerol + carboxel L.V. 3%	Carboxel H.V. 3%	Carboxel L.V. 3%
Substrate: benzyl-n-butyrate						
4	297	505	725	794	753	706
14	270	358	655	599	580	519
40	150	210	627	510	541	510
Substrate: ethyl-n-butyrate						
4	307	358	1050	1089	1050	900
14	144	275	978	1005	1010	810
40	65	250	715	790	965	800
Substrate: tributyrin						
4	514	576	975	1146	1010	976
14	288	307	923	1033	949	860
40	285	302	920	890	620	730
Substrate: olive oil						
4	266	558	367	498	290	357
14	74	248	266	283	266	250
40	74	110	220	260	225	150
Substrate: emulsion of tubercle bacilli						
4	278	416	378	530	307	369
14	76	157	373	490	205	275
40	23	120	240	365	155	269

(4). Gum acacia added in the same proportion to the *extracts of L.G.M.* activated the cleavage of all the substrates, especially of benzyl butyrate, olive oil, beeswax, and the lipoids of the tubercle bacilli.

The following conclusions may be drawn from these observations:

(1). The lipidolytic enzyme system of L.G.M. contains lipases that are absent in beef pancreas, and that have a specific action on wax and waxy compounds.

(2). Biochemically, the specific action of L.G.M. on these substrates can be defined as an affinity for lipids formed by high alcohols with lower fatty acid chains.

In a more general way the findings suggest that the specificity of action of any lipase is determined by the alcohols of the lipid substrate rather than by the nature of the fatty acid chains.

(3). The difference in fat splitting enzymes of pancreas and L.G.M. is amplified by the activating or inhibiting properties of emulsifiers.

After thus establishing the special nature of the enzymes of L.G.M., "Carboxel" was found to be an even better emulsifying agent than those previously used. "Carboxel" is found on the market (Standard Chemical Company Ltd.) as a powder of high viscosity (HV) and low viscosity (LV). This chemical, used as emulsifier, activates the fat splitting process. Furthermore in 1.5% concentrations in 50% glycerol for the extraction of the L.G.M. powder, it retarded the deterioration of the enzyme in the extract. The best results so far obtained in regard to extraction and preservation of the lipases acting on high alcohol – low fatty acid compounds were when the L.G.M. powder was emulsified for three days at room temperature (pH 7.2) in Carboxel–glycerol, and the filtrates were kept in the Frigidaire. . The glycerol–Carboxel extract of L.G.M. powder was more than twice as active on tributyrin as the glycerol–extract. While, 14 days after extraction, the lipidolytic activity of the glycerol–extract on tributyrin was diminished by nearly 50%, that of the glycerol–Carboxel extract was diminished by 10%. While in two weeks the glycerol extract lost around 75% of its activity on tubercle bacilli emulsion, the Carboxel–glycerol extract showed no appreciable loss of activity over the same period.

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THE RELATIONSHIP OF SERUM PHOSPHATASES TO SEX HORMONES[1]

BY JULES TUBA, DONALD B. BAKER, AND MAX M. CANTOR

Abstract

Castration produces a fall in serum inorganic phosphorus values in adult male rats but not in adult female rats. The alterations of phosphorus levels that occur following castration in male rats and after the injection of some hormones into normal and castrated rats of both sexes may be accounted for by corresponding alterations in metabolic requirements. Neither castration nor hormone injection produces any significant change in serum acid phosphatase activity in male or in female rats. A fall of about 30% to levels approaching values for normal female rats is found in serum alkaline phosphatase of castrated male rats in about eight weeks. There is no change in the enzyme values in castrated females. In those instances where injections of a sex hormone into castrated or normal rats produce alterations in alkaline serum phosphatase values such changes may be accounted for on the basis of altered food intake. The sole exception to this finding is the very marked decrease in the activity of the enzyme produced by progesterone after oestrogen in normal male rats.

Introduction

The investigations of the Gutmans (5, 7, 9), and of Huggins and Hodges (6) have demonstrated the importance of determining levels of serum "acid" and "alkaline" phosphatases both in the diagnosis of metastasizing prostatic carcinomas and following therapy with oestrogens or castration.

Observations on the effects of sex hormones on serum phosphatases have been carried out with experimental animals by Huggins and Hodges (6), and by Buchwald and Hudson (1, 2), but the results are scanty and conflicting. The investigations reported in this paper were carried out in a manner that controls a number of variable factors not usually considered and that can markedly affect the experimental data.

Experimental

Male and female castrated and intact adult albino rats (Wistar strain) were used. The castrated animals were left for an eight week postoperative period before being used for hormone injections.

During the development of the investigation it was found that in order to obtain normal mean values for serum alkaline phosphatase in intact rats it was necessary to consider the hour of day at which the animals were bled and the amount and type of food ingested. It was found that the levels of the alkaline enzyme dropped during the daylight hours and rose during the evening and night to reach a peak about 9 a.m. This diurnal variation is shown graphically in Fig. 1. It was also observed that if the animals were not fed their full daily requirements of approximately 27 gm. per day of Purina Fox Checkers their alkaline phosphatase levels were lower than those fed

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[1] *Manuscript received January 20, 1949.*
Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta.

ad libitum. Subsequently Cantor, Wight, and Tuba (3) were able to show conclusively that it was the decrease in total daily fat ingestion, rather than the lowered calorie intake, that caused the lowering of the alkaline enzyme.

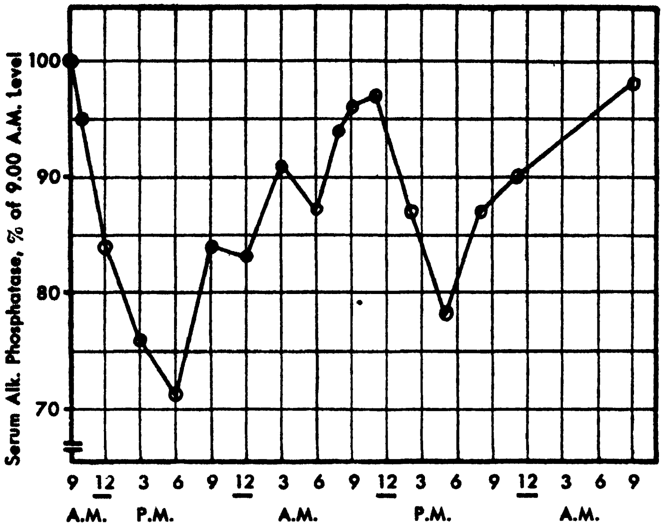

FIG. 1. *The daily variation in the serum alkaline phosphatase of adult male rats receiving adequate food.*

It was felt that the serum phosphatases of the female rat might be affected by the fluctuating hormone levels associated with various stages of the oestrous cycle. However, examination of a group of 30 adult females indicated that there was no significant alteration in levels of serum phosphorus or phosphatases of animals shown by vaginal smears to be in oestrus.

In view of the above findings, it was decided to feed the animals in excess of their requirements and to bleed them at 10 a.m. Using this regime, normal values for serum inorganic phosphorus, and for serum acid and alkaline phosphatases were established by means of the Shinowara, Jones, and Reinhart (8) micromethod as modified by Gould and Schwachman (4), and these are given in Tables I and II. It is important to note that the mean normal value of alkaline serum phosphatase for adult male rats is 113 units per 100 ml. of serum, and that this is markedly higher than the normal mean value for adult female rats of 70 units per 100 ml. serum.

In order to determine the effect of castration on serum inorganic phosphorus and phosphatases these were estimated once a week postoperatively for 8 to 12 weeks. Table III shows that in castrated male rats the serum alkaline phosphatase dropped about 30% from the normal level toward female values in approximately eight weeks. Beyond this time the alkaline phosphatase was found to fluctuate between 65 and 100 units, and the inorganic phosphorus decreased to lower than normal values. The acid enzyme showed no significant alterations.

TABLE I

THE MEAN, STANDARD ERROR OF THE MEAN, STANDARD DEVIATION, AND THE RANGE FOR SERUM INORGANIC PHOSPHORUS AND SERUM ACID AND ALKALINE PHOSPHATASES IN NORMAL MALE ADULT RATS

	Number of rats	Mean	Standard error of mean × 2	Standard deviation	Range
Serum inorganic phosphorus	141	8.0 mgm./100 ml.	± 0.2 mgm./100 ml.	± 1.2 mgm./100 ml.	5.4–12.4 mgm./100 ml.
Serum acid phosphatase	130	2.6 units/100 ml.	± 0.2 units/100 ml.	± 1.1 units/100 ml.	0.3–5.2 units/100 ml.
Serum alkaline phosphatase	151	113 units/100 ml.	± 5 units/100 ml.	± 28 units/100 ml.	35–189 units/100 ml.

TABLE II

THE MEAN, STANDARD ERROR OF THE MEAN, STANDARD DEVIATION, AND THE RANGE FOR SERUM INORGANIC PHOSPHORUS AND SERUM ACID AND ALKALINE PHOSPHATASES IN NORMAL FEMALE ADULT RATS

	Number of rats	Mean	Standard error of mean × 2	Standard deviation	Range
Serum inorganic phosphorus	120	7.2 mgm./100 ml.	± 0.3 mgm./100 ml.	± 1.5 mgm./100 ml.	4.7–15.5 mgm./100 ml.
Serum acid phosphatase	120	2.6 units/100 ml.	± 0.2 units/100 ml.	± 0.9 units/100 ml.	0.0–5.4 units/100 ml.
Serum alkaline phosphatase	122	70 units/100 ml.	± 4 units/100 ml.	± 21 units/100 ml.	34–128 units/100 ml.

TABLE III

THE EFFECT OF ORCHIDECTOMY ON SERUM PHOSPHATASES AND INORGANIC PHOSPHORUS. (MEAN OBTAINED FROM 10 RATS)

Number of weeks after operation	Serum inorganic phosphorus, mgm./100 ml.	Serum acid phosphatase, units/100 ml.	Serum alkaline phosphatase, units/100 ml.
Preoperative	8.0	2.3	106
1	8.2	3.5	103
2	8.5	2.4	96
3	8.4	2.6	96
4	8.1	4.2	95
5	8.2	2.8	80
6	8.1	3.7	72
7	8.0	1.0	69
8	7.9	2.5	63
10	7.2	3.2	85
12	5.8	2.0	96

In Table IV is shown the effect of öophorectomy on the serum phosphatases and phosphorus. The alterations here are essentially negative.

TABLE IV

THE EFFECT OF ÖOPHORECTOMY ON SERUM PHOSPHATASES AND INORGANIC PHOSPHORUS. (MEAN OBTAINED FROM 10 RATS)

Number of weeks after operation	Serum inorganic phosphorus, mgm./100 ml.	Serum acid phosphatase, units/100 ml.	Serum alkaline phosphatase, units/100 ml.
Preoperative	6.2	3.1	69
1	6.8	2.6	61
2	6.4	2.0	63
3	5.9	1.9	57
4	5.8	3.1	90
5	5.9	5.3	86
6	5.6	5.4	77
7	6.8	1.9	80
8	6.0	3.0	70

The sex hormones that were used to determine their effect on serum inorganic phosphorus and phosphatases of male and female rats were testosterone propionate, oestradiol dipropionate, diethylstilboestrol, progesterone, progesterone after oestradiol priming, and desoxycorticosterone acetate. Each hormone was tested on castrated animals and on intact animals. Groups of 10 rats were used for each test. The hormones, dissolved in oil, were injected intramuscularly in the dosages and at the time intervals shown in Tables V and VI. Desoxycorticosterone acetate, while not properly a sex hormone, was included because of its steroid nature.

Table V shows that significant changes are produced in the serum inorganic phosphorus level of male rats by some of the hormones. Testosterone injections caused a marked elevation of the typically low phosphorus levels of the castrates, but had no effect on normal males. Oestradiol, as well as diethylstilboestrol, produced a lowering of the phosphorus values in both normal and castrated male animals. Progesterone lowered the phosphorus in the serum of normal males and elevated the values in castrates. Injection of oestradiol on the second and fourth days of the experiment, followed by six daily injections of progesterone beginning the seventh day, produced a pronounced fall in normal male serum phosphorus similar to the effect of progesterone alone. Serum acid phosphatase results may be considered to be negative. Serum alkaline phosphatase values are significantly decreased by the female hormones in several instances. Oestradiol caused a decrease in castrates and normal animals. Diethylstilboestrol, progesterone, and progesterone after oestradiol produced lowered serum alkaline phosphatase activity in normal male rats.

Table VI indicates the changes occurring in the phosphorus levels in the serum of female rats. Testosterone and oestradiol increase the phosphorus

TABLE V

THE EFFECT OF SEX HORMONES ON THE SERUM PHOSPHATASES AND INORGANIC PHOSPHORUS OF ADULT MALE RATS. (EACH RESULT THE MEAN OF 10 RATS)

Sex hormone dosage	Number of days after initial injection	Number of injections	Serum inorganic phosphorus, mgm./100 ml.		Serum acid phosphatase, units/100 ml.		Serum alkaline phosphatase, units/100 ml.	
			Cast. males	Normal males	Cast. males	Normal males	Cast. males	Normal males
Testosterone propionate, 1 mgm./day/rat	0	0	5.1	8.0	1.6	2.1	70	97
	15	14	10.8	7.8	0.5	4.3	62	75
	31	21	7.1	7.5	1.6	3.0	85	102
Oestradiol dipropionate, 0.1 mgm./day/rat	0	0	6.3	8.5	2.4	3.1	98	114
	5	4	7.0	8.5	1.9	3.2	61	76
	9	6	5.9	6.2	1.2	3.8	40	61
Diethylstilboestrol, 0.1 mgm./day/rat	0	0	6.5	8.8	1.8	2.4	58	122
	5	4	5.8	8.5	2.4	3.9	49	94
	9	6	5.6	6.8	3.3	4.0	68	82
Progesterone, 1 mgm./day/rat	0	0	5.7	10.1	2.4	3.1	76	115
	7	6	5.2	7.4	1.5	3.7	55	74
	14	12	8.9	6.5	1.9	4.5	66	66
Progesterone following oestradiol dipropionate,	0	0	5.4	10.3	3.1	3.1	90	102
0.1 mgm. oest./day/rat	7	2 oest.	4.5	8.4	1.9	2.3	42	39
1 mgm. prog./day/rat	14	6 prog.	5.5	6.6	2.0	3.6	59	31
Desoxycorticosterone acetate, 0.2 mgm./day/rat	0	0	5.7	7.0	2.5	1.9	81	90
	7	6	6.5	6.2	1.4	2.4	70	82
Sesame oil, 0.2 ml./day/rat	0	0	5.9	8.8	2.1	2.4	83	116
	14	13	6.0	9.3	—	—	72	85

TABLE VI

THE EFFECT OF SEX HORMONES ON THE SERUM PHOSPHATASES AND INORGANIC PHOSPHORUS OF ADULT FEMALE RATS. (EACH RESULT THE MEAN OF 10 RATS)

Sex hormone dosage	Number of days after initial injection	Number of injections	Serum inorganic phosphorus, mgm./100 ml.		Serum acid phosphatase, units/100 ml.		Serum alkaline phosphatase, units/100 ml.	
			Cast. females	Normal females	Cast. females	Normal females	Cast. females	Normal females
Testosterone propionate, 1 mgm./day/rat	0	0	4.9	7.9	2.3	2.5	78	83
	14	13	6.5	8.9	1.9	1.6	45	76
Oestradiol dipropionate, 0.1 mgm./day/rat	0	0	5.8	7.0	3.5	2.4	62	71
	7	6	6.8	8.6	2.2	2.1	58	63
Diethylstilboestrol, 0.1 mgm./day/rat	0	0	5.8	5.8	2.4	2.0	54	61
	7	6	6.3	7.3	2.6	1.8	26	43
Progesterone, 1 mgm./day/rat	0	0	6.5	7.2	5.0	3.5	76	67
	14	13	7.0	8.5	1.4	2.7	46	64
Desoxycorticosterone acetate, 0.2 mgm./day/rat	0	0	6.9	7.1	3.1	2.7	85	86
	7	6	7.2	7.0	2.4	2.6	47	50

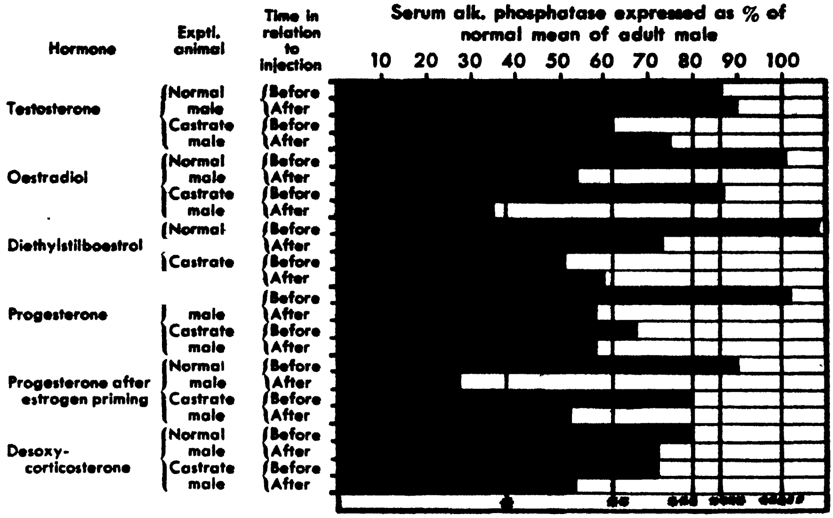

FIG. 2. *Serum alkaline phosphatase variations in the adult male rat resulting from the injection of sex hormones.*†

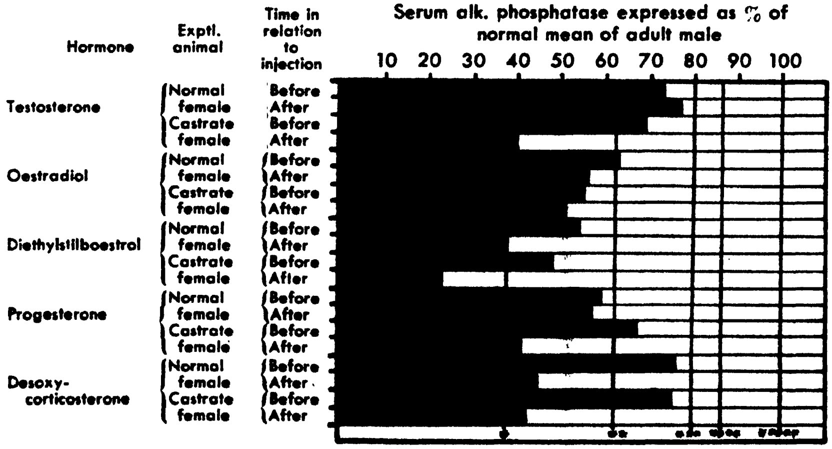

FIG. 3. *Serum alkaline phosphatase variations in the adult female rat resulting from the injection of sex hormones.*†

† NOTE: *Limiting values in Figs. 2 and 3 are calculated from the data of Tables I and II.*

* *Limit below which alterations from mean for normal female rats are significant.*

** *Mean for normal female rats.*

*** *Limit below which alterations from mean for normal male rats are significant.*

**** *Limit above which alterations from mean for normal female rats are significant.*

***** *Mean for normal male rats.*

levels in castrates and in normal rats. Diethylstilboestrol as also progesterone increases the values in the sera of normal female rats. Serum acid phosphatase changes are essentially negative. The only statistically significant fall in alkaline phosphatase is found in castrated females following injections of diethylstilboestrol.

Figs. 2 and 3 illustrate graphically the data given in Tables V and VI.

In order to determine whether the results obtained by hormone injections were due to the hormone per se or due to the amount of food ingested, a record was kept of the daily amounts of food eaten by the animals under various experimental conditions, and Table VII indicates these values.

TABLE VII

DAILY AVERAGE FOOD INTAKE OF ADULT RATS UNDER VARIOUS EXPERIMENTAL CONDITIONS REFERRED TO IN TABLES V AND VI

Condition of the experimental animal	Hormone injected	Amount of food ingested/rat/day in grams
Normal male		27
Normal female		15
Castrated male		Decreases from 27 gm. at time of operation to 15 gm. 8 weeks later. Marked fluctuations then occur
Castrated female		15
Normal males	Testosterone	23
	Oestradiol	10
	Diethylstilboestrol	10
	Progesterone	21
	Oestradiol and progesterone	10 during first week increasing to 20 in second
	Desoxycorticosterone	19
Castrated males	Testosterone	24
	Oestradiol	10
	Diethylstilboestrol	10
	Progesterone	20
	Oestradiol and progesterone	10 during first week increasing to 20 in second
	Desoxycorticosterone	11
Normal females	Testosterone	17
	Oestradiol	14
	Diethylstilboestrol	6
	Progesterone	15
	Desoxycorticosterone	15
Castrated females	Testosterone	11
	Oestradiol	8
	Diethylstilboestrol	4
	Progesterone	13
	Desoxycorticosterone	13

Attempts were then made to correlate the amount eaten by the animals with the levels of serum alkaline phosphatase, and the coefficient of correlation was calculated and found to be 0.70. This indicates that variations occurring in serum alkaline phosphatase due to castration or injection of sex hormones could be accounted for by an altered food intake. The one notable exception to this is the profound lowering of the alkaline enzyme level produced by progesterone after oestrogen priming in normal adult males.

Acknowledgments

These investigations were carried out with the assistance of a research fellowship granted by the College of Physicians and Surgeons of Alberta to Donald B. Baker. The authors are indebted to Merck and Co. for sodium β-glycerophosphate and to Ciba Co. Ltd., for the various hormones used in the work reported above.

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 27, SEC. E. AUGUST, 1949 NUMBER 4

BLOOD AND SPINAL FLUID GUANIDINE IN EPILEPSY[1]

BY K. A. C. ELLIOTT AND NORA HENDERSON

Abstract

It has been reported that the amount of guanidine-like substances in the blood of "essential" epileptics is higher than the normal and increases considerably before and during seizures. In the present study no sign of a raised "guanidine" level was found in the blood or spinal fluid or any type of epileptic patient.

Murray and Hoffman (3) reported that in 15 cases of "essential epilepsy" the basal level of substances estimated as guanidine in the blood was higher than in normal subjects and that it increased during the aura, reached a considerably higher value during a convulsion, and was still somewhat above the basal level, one hour after the convulsion. The averages of all the figures given, in mgm. per 100 ml., were 0.41 basal, 0.70 aura, 1.23 in convulsion, and 0.54 one hour postconvulsion, but there was considerable individual variation.

Guanidine is a convulsant and some of its pharmacological effects resemble those of eserine. It is closely related to creatine and phosphocreatine, which are intimately concerned in the chemical dynamics of nervous and other tissues. The guanidine group is also part of the purine and pyrimidine nuclei, which constitute part of a number of important coenzymes as well as nucleic acid derivatives. The finding of an increase in guanidine-like substances in relation to epilepsy would therefore be an important clue to the nature of the epileptic process.

Palmer, Scott, and Elliott (4), who were interested in the possible relation of migraine to epilepsy, found no significant differences between the average blood "guanidine" levels of normal subjects and migraine patients (during or between attacks), epileptics (interictal), and various other patients. The value obtained was about the same as the basal value for epileptics given by Murray and Hoffmann. The latter authors gave no values themselves for normal subjects but accepted the value of 0.21 to 0.28 given by Andes and Myers (1). Palmer *et al.* concluded that the average basal value for epileptics was not different from normal but that the similar modifications of the analytical method used by themselves and by Murray and Hoffmann gave slightly higher values than the original method of Andes and Myers.

[1] *Manuscript received March 26, 1949.*

Contribution from the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal, Que.

TABLE I

GUANIDINE-LIKE SUBSTANCES IN BLOOD AND SPINAL FLUID OF EPILEPTIC PATIENTS

Patient	Type, and time since, last seizure	"Guanidine" level mgm. per 100 ml.	
		Blood	Spinal fluid
R. L. Encephalopathy	General, 3.5 hr.	0.22	
M. W. Postencephalitis	General, 1 day	0.24	
M. B. Encephalopathy	In status epilepticus	0.51	
M. F. Neoplasm?	In status epilepticus	0.24	0.07
E. P. Birth injury	Continuous twitches*	0.25	0.10
J. H. " "	General, during seizure	0.42	
N. A. Focal epilepsy; cause unknown	General, 15 min.	0.25	0.10
C. G. Idiopathic epilepsy	General, 9 days	0.40	
J. T. " "	General, 6 days	0.47	
G. K. " "	General, 26 min., and petit mal every few min.	0.61	0.17
H. S. " "	General, 10 min.	0.39	
R. S. " "	General, 10 min.	0.37	
C. E. " "	General, 5 min.	0.50	
W. L. " "	General, during seizure	0.26	
J. M. " "	Petit mal,* 5 min.	0.19	
S. P. " "	Petit mal, 5 min.	0.35	
J. L. " "	Petit mal, 3 min.	0.36	
E. S. " "	Petit mal,* 1 min.	0.40	0.10
L. D. " "	Petit mal, 0 min.	0.26	
G. K. " "	Petit mal every few min.	0.59	
G. K. " "	Petit mal every few min.	0.43	0.15
Metrazol provoked seizures			
M. W. Postencephalitis	General, 4 min.	0.26	
Y. B. Idiopathic epilepsy	General, 0 min.	0.40	

* *Patient also had generalized seizures.*

In the present study, determinations have been made of substances estimated as guanidine in the blood and spinal fluid from a number of different types of patients showing epileptic symptoms.

The analytical method, a slight modification of the method of Andes and Myers (1), was the same as that used by Palmer *et al.* except that, in all but three cases (M.B., R.S., W.L.), deproteinization was achieved with trichloroacetic acid instead of tungstic acid. As soon as possible after withdrawal, one volume of blood or of spinal fluid was run into four volumes of 10% trichloroacetic acid and the analysis was carried out on 25 ml. of the filtrate. This was neutralized to about pH 7 with *N* sodium hydroxide, using small pieces of "Alkacid paper" to test the reaction, before carrying on the usual procedure. Tests showed that methyl guanidine added to the blood was about 70% recovered after either method of protein precipitation but that the basal values were about 0.14 mgm. % lower after trichloroacetic acid than after tungstic acid treatment. The results given here (except for the above-mentioned three) should be increased by about this amount to make them comparable with those of Palmer *et al.* and Murray and Hoffmann. Since virtually the same method was used as previously (Palmer *et al.*) only three new determinations on normal blood were made; the figures obtained were 0.46, 0.29, 0.25.

The results obtained with blood and spinal fluid from epileptic patients are shown in Table I. In no case was a strikingly high value for blood "guanidine" obtained even when the blood was drawn during or very shortly after a seizure. The figures are generally within the normal range. Seizures induced in two epileptic patients by the slow administration of metrazol (2) did not give rise to high "guanidine" levels. In a few cases determinations were made on spinal fluid, drawn at the same time as the blood. The values obtained were low in all cases, and near the limit of sensitivity of the method.

There is thus no suggestion from this series of patients that the blood or spinal fluid "guanidine" level is affected in epileptic conditions. No explanation can be offered for the difference between these results and those of Murray and Hoffmann.

Acknowledgment

We are glad to acknowledge the co-operation and advice of Dr. D. McEachern and the Neurological Service of this Institute. We are grateful also to Dr. H. Lehmann and the staff of the Verdun Protestant Hospital for the provision of a number of blood samples.

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ON THE ABO GENE AND Rh CHROMOSOME DISTRIBUTION IN THE WHITE POPULATION OF MANITOBA[1]

By B. Chown, R. F. Peterson, Marion Lewis, and Ann Hall

Abstract

In the Province of Manitoba, Canada, all men and women prior to marriage have their ABO and Rh blood groups determined. An analysis has been made of ABO and Rh data from blood samples of 3100 individuals of white race.

The calculated O, A, B gene frequencies in per cent based on the sample of 3100 individuals were as follows: O, 65.77; A, 26.07; B, 8.48. The calculated frequencies of Rh chromosomes for the same sample in per cent were as follows: R_1, 43.48; r, 39.55; R_2, 12.87; R_0, 1.91; R′, 1.24; R″, 0.73; R_z, 0.22; R_y 0.00.

Frequencies of O, A, B genes and Rh chromosomes within the major national groups were also estimated and shown in comparison with the corresponding O, A, B gene frequencies for Great Britain and Rh chromosome frequencies for England.

The major national groups were compared in pairs by means of the Test of Homogeneity, with respect to the distribution, first of the ABO phenotypes, and then of the major Rh phenotypes. In general the Hebrew, Ukrainian, and Polish differed significantly from the remaining groups in distribution of the ABO phenotypes, due to a comparatively high frequency of the B phenotype in the former. With respect to the distribution of the Rh phenotypes the Hebrew group differed significantly from all others, due to a comparatively low incidence of the r phenotype.

Introduction

The previous studies in Manitoba (2, 3) were concerned with the Rh types in Canadians of Japanese race who had come to Manitoba and Western Ontario from British Columbia in 1942.

The purpose of the present paper is to study the distribution of the ABO genes and the Rh chromosomes in the white population of Manitoba through an analysis of data on 3100 individuals.

Material

A. Blood Samples

In the Province of Manitoba, Canada, every man and woman prior to marriage must, by law, have a serological test for evidence of syphilis done in the central laboratory of the Provincial Department of Health. The specimens of blood for this purpose are collected in sterile vacuum tubes, and after arrival at the laboratory are kept refrigerated. When that laboratory has finished with the blood, it is turned over to us. The specimens are, therefore, from two to seven days old when we receive them, but as they have been handled with care, and have been kept refrigerated, they reach us in good condition.

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[1] *Manuscript received in original form January 18, 1949, and, as revised, May 7, 1949.*
Contribution from the Children's Hospital, the Dominion Laboratory of Cereal Breeding, and the Department of Paediatrics, Faculty of Medicine, University of Manitoba, Winnipeg, Man.

B. Sera

(1) Anti-A and anti-B.* These were prepared from donors with high titer, high avidity sera. Their antibody titer was not increased by artificial means.

(2) Anti-Rh. The following sera were used:

Anti-C. A single serum of our own, giving a strong reaction with saline suspended cells and having a titer of 1 : 32. Whether this serum is pure anti-C or is anti-C + C^w is not known. It contains an anti-D that agglutinates D-positive cells in albumen suspension but not in saline.

Anti-D. All bloods were set up against three strong anti-D sera. All those that were negative to these three and positive to either anti-C or anti-E or both were checked with two further anti-D sera. Undoubtedly even with this, some bloods that contained variants of Stratton's D^u antigen (7) were classed as D-negative since, as Race, Sanger, and Lawler (6) have reported, the elimination of all D^u bloods from apparent D-negatives requires "titrations in saline and in albumen against numerous anti-D sera, and tests by the anti-globulin and by the elution techniques". However, the error is probably small, less than 1% (6).

Anti-E. A single serum was used.**

Anti-c. A single serum was used.**

Methods

Agglutinogens A and B and agglutinins anti-A and anti-B were demonstrated by the open-well slide technique, both agglutinogen and agglutinin content of all bloods being determined. The Rh agglutinogens were demonstrated by our capillary method (1).

Results

Table I is presented in order to indicate the proportions of the various "races" or national groups making up the population of Manitoba. The data are from the 1941 Census as given in the Canada Year Book for 1945, to which we have added a column showing the percentage of each "race".

The data on which the present study is based are shown in Tables II and III. The number of individuals of each ABO and Rh phenotype are shown for each reported "racial origin" group. The latter term requires explanation.

In the official notice of marriage in the Province, from which notice our data on "racial origin" are drawn, brides and grooms are required to state their

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* *The blood groups specific substances A and B were kindly provided by Sharpe and Dohme of Philadelphia.*

** *These sera were generously supplied by Dr. Louis K. Diamond, Boston.*

TABLE I

RACIAL ORIGINS OF THE POPULATION OF MANITOBA, 1941
(CANADA YEAR BOOK, 1945)

Racial origin	Number of individuals	Percentage
British Isles Races	360,560	49.41
English	168,917	23.15
Irish	76,156	10.44
Scottish	109,619	15.02
Other	5868	0.80
Other European Races	342,393	46.92
French	52,996	7.26
Austrian	4719	0.65
Belgian	6715	0.92
Bulgarian	24	0.003
Czech and Slovak	3702	0.51
Danish	3164	0.43
Finnish	808	0.11
German	41,479	5.68
Greek	399	0.05
Hungarian	2418	0.33
Icelandic	13,954	1.91
Italian	2482	0.34
Jewish	18,879	2.59
Lithuanian	407	0.06
Netherlands	39,204	5.37
Norwegian	5955	0.82
Polish	36,550	5.01
Rumanian	1438	0.20
Russian	6571	0.90
Swedish	9547	1.31
Ukrainian	89,762	12.30
Yugoslavic	451	0.06
Other	769	0.11
Asiatic Races	1788	0.24
Chinese	1248	0.17
Japanese	42	0.006
Other	498	0.07
Eskimo	1	0.0001
Indian	15,473	2.12
Negro	453	0.06
Other	8796	1.20
Not stated	280	0.04
	729,744	

"racial origin". No explanation of this term is given in the form itself, but as will be evident from Tables II and III, the term "race" is used mainly in a national sense. The term "Canadian" is not accepted in these reports, each individual being required to trace his ancestry to some other nationality or race. This is customarily done through the male line. Thus, for example, if an individual's paternal grandfather were English and the other three

TABLE II

DISTRIBUTION OF 3100 INDIVIDUALS OF WHITE RACE WITH RESPECT TO ABO PHENOTYPES

"Racial origin"	ABO phenotypes				Total
	O	A	B	AB	
American	1	1			2
Armenian		1			1
Austrian	5	13	2	1	21
Belgian	15	20			35
Czech	3	6	2	1	12
Danish	6	7		1	14
Dutch	52	49	6	6	113
English	372	331	71	18	792
Estonian		1			1
Finnish	2	1	1		4
Flemish			1		1
French	117	119	19	8	263
German	73	63	12	7	155
Greek	2	1			3
Hebrew	48	62	24	6	140
Hungarian	2	2		1	5
Icelandic	33	23	7	1	64
Irish	147	119	39	11	316
Italian	4	5	2	2	13
Latvian	2	1			3
Lithuanian			1		1
Norwegian	12	10	3	3	28
Polish	40	47	21	10	118
Rumanian	4	1		1	6
Russian	10	15	6	1	32
Ruthenian	2				2
Scottish	244	188	55	15	502
Spanish	1	1			2
Swiss	2	2			4
Swedish	21	25	7	3	56
Syrian	2		1	1	4
Ukrainian	108	141	82	19	350
Welsh	9	17	5	1	32
Yugoslav	2	2	1		5
Totals	1341	1274	368	117	3100

grandparents were Scotch, French, and German, respectively, the individual in question would be expected to report his "racial origin" as being English. This is also the procedure followed in making census returns.

We considered that it would be of interest not only to analyze the data from the sample of 3100 individuals as a whole but also to deal with some of the larger "racial" groups separately. We would caution the reader to keep in mind, however, the indefinite and rather artificial basis of the determination of these groups.

In order to have some measure of the extent to which our sample is representative of the Manitoba white population as a whole, we have attempted to compare the data on racial origins obtained from the marriage records of the 3100 individuals with those of the 1941 census as shown in Table I. However, the census figures do not show the proportion of whites in the second last

TABLE III

DISTRIBUTION OF 3100 INDIVIDUALS OF WHITE RACE WITH RESPECT TO Rh PHENOTYPE

"Racial origin"	Rh phenotypes and reactions to antisera C, D, E, and c											Total
	R_1r ++−+	R_1 ++−−	r −−−+	R_2 −+++	R_1R_2 ++++	R_0 −+−+	R′r +−−+	R″ −−++	R_z +++−	R′ +−−−	R′R″ +−++	
American	2											2
Armenian	1											1
Austrian	10	4	2	1	3	1						21
Belgian	9	6	9	6	5							35
Czech	4	3	4		1							12
Danish	5	3	2	2	2							14
Dutch	41	23	17	13	17	2						113
English	265	140	134	117	107	13	6	7	1	2		792
Estonian					1							1
Finnish	1	2		1								4
Flemish				1								1
French	79	52	36	38	49	3	2	3			1	263
German	43	29	26	25	26	1	2	2		1		155
Greek	3											3
Hebrew	49	41	11	7	24	5	2		1			140
Hungarian	1		1	3								5
Icelandic	15	17	6	11	12	1	1	1				64
Irish	108	60	56	46	39	3	3	1				316
Italian	5	4		1	2				1			13
Latvian	1	2										3
Lithuanian	1											1
Norwegian	6	9	1	5	7							28
Polish	33	30	16	16	21	1	1					118
Rumanian	2	2			2							6
Russian	12	5	4	4	6	1						32
Ruthenian	1				1							2
Scottish	171	86	81	85	66	6	2	3	2			502
Spanish	2											2
Swiss		1		2	1							4
Swedish	12	13	9	10	8	2	1	1				56
Syrian	1	1	1			1						4
Ukrainian	130	57	65	47	37	6	7		1			350
Welsh	13	2	2	6	6	2	1					32
Yugoslav	2		2	1								5
Total	1028	592	485	448	443	48	28	18	6	3	1	3100

TE: *We assumed the absence of chromosome Ry in our population, since its existence had not been proved at the time this was written. Proof of its existence has since been established by Van den Bosch (Nature, 162 : 781-783, 1948). Since its frequency is probably less than 0.005 its omission does not materially affect our calculations.*

group of Table I designated as "Other", nor in the Asiatic races designated as "Other", and there is an unidentified group of 280 persons at the bottom of the table. For these reasons we have compiled Table IV to include peoples of European origin only. This excludes the four Syrians and the one Armenian from our sample. The Hebrews of Manitoba are of European ancestry, although the original source of this race is Asia.

TABLE IV

NUMBER OF WHITE INDIVIDUALS OF EUROPEAN ORIGIN IN MANITOBA

"Racial origin"	1941 Census		Sample used in present study	
	Number	Percentage	Number	Percentage
English	168,917	24.03	792	25.59
Irish	76,156	10.83	316	10.21
Scottish	109,619	15.59	502	16.22
Other British Isles Races	5868	0.83	32	1.03
French	52,996	7.54	263	8.50
Austrian	4719	0.67	21	0.68
Belgian	6715	0.96	35	1.13
Czech and Slovak	3702	0.53	12	0.39
Danish	3164	0.45	14	0.45
Finnish	808	0.11	4	0.13
German	41,479	5.90	155	5.01
Greek	399	0.06	3	0.10
Hungarian	2418	0.34	5	0.16
Icelandic	13,954	1.98	64	2.07
Italian	2482	0.35	13	0.42
Jewish	18,879	2.69	140	4.52
Lithuanian	407	0.06	1	0.03
Netherlands	39,204	5.58	113	3.65
Norwegian	5955	0.85	28	0.90
Polish	36,550	5.20	118	3.81
Rumanian	1438	0.20	6	0.19
Russian	6571	0.93	32	1.03
Swedish	9547	1.36	56	1.81
Ukrainian	89,762	12.77	350	11.31
Yugoslavic	451	0.06	5	0.16
Other European Races	793	0.11	15	0.48
Total European	702,953		3095	

The corresponding percentages shown in Table IV for the 1941 census and for our sample are strikingly similar, but when the Test of Homogeneity as described by Fisher (4) is applied to the data of Table IV (using the absolute numbers, not percentages) a χ^2 value of 143.779 is obtained with 25 degrees of freedom, indicating a statistically significant difference between our sample and the 1941 population with respect to the distribution of individuals in the designated racial groups.

The difference between the two series appears to be small from a biologic standpoint, but statistically significant. When large numbers, such as we have here, are available for analysis, it is possible to demonstrate the statistical significance of quite small differences. Such a difference as has been demonstrated here is not surprising since the two sets of data were taken at two different times and one set was obtained from a restricted age group.

Estimation of Gene Frequencies

The following formulae were used for calculating ABO gene frequencies:

$$O = \sqrt{(O)}$$

$$A = \sqrt{(O) + (A)} - \sqrt{(O)}$$

$$B = \sqrt{(O) + (B)} - \sqrt{(O)}$$

where O, A, and B represent gene frequencies and (O), (A), (B) represent phenotype frequencies.

For the calculation of frequencies of Rh chromosomes we have used the formulae provided by Race, Mourant, and McFarlane (5).

These are as follows:—

$$r = \sqrt{(---+)}$$

$$R' = \sqrt{(---+) + (+--+) + (+---)} - \sqrt{(---+)}$$

$$R'' = \sqrt{(---+) + (--++)} - \sqrt{(---+)}$$

$$R_0 = \sqrt{(---+) + (-+-+)} - \sqrt{(---+)}$$

$$R_1 = \sqrt{(++--) + (+--+)} - R'$$

$$R_z = \sqrt{(++--) + (+--+) + (+++-)} - (R_1 + R')$$

$$R_2 = 1 - (r + R' + R'' + R_0 + R_1 + R_z)$$

where the frequency of phenotype $---+$ (see Table III) is represented by $(---+)$.

The calculations were made on the entire sample of 3100 individuals and also on separate national groups having 100 or more individuals in the sample. The estimates of frequencies of OAB genes are presented in Table V, and the Rh chromosome frequencies in Table VI.

The most striking feature of Table V is the higher incidence of the gene B in Ukrainian, Polish, and Hebrew groups as compared to the other groups. Our estimates of O, A, and B gene frequencies based on the combined English, Scottish, and Welsh groups agree very closely with corresponding estimates for Great Britain made by Dobson and Ikin, and based on members of the Royal Air Force. The latter set of data was considered to be representative of Great Britain as a whole.

TABLE V

Estimated frequencies of the genes O, A, and B in Manitoba whites in comparison with data from Great Britain

"Racial origin"	Number in sample	Frequency, %		
		O	A	B
English	792	68.53	25.68	6.25
Scottish	502	69.72	23.05	7.46
Irish	316	68.20	23.54	8.52
German	155	68.63	25.04	5.43
Dutch	113	67.84	26.70	3.81
French	263	66.70	28.03	5.21
Ukrainian	350	55.55	28.80	18.13
Polish	118	58.22	27.64	13.68
Hebrew	140	58.55	30.09	13.16
Others	351	63.16	28.52	8.26
Total	3100	65.77	26.07	8.48
Present study: English, Scottish, and Welsh	1326	68.65	24.92	6.85
Data from Great Britain*	190,177	68.31	25.69	6.00

* *Aileen M. Dobson and Elizabeth W. Ikin. The ABO blood groups in the United Kingdom. J. Path. Bact. 58 : 221-227. 1946.*

In Table VI the Rh chromosomes appear to have fairly comparable distributions in the various groups except for the Hebrew group, in which there is a comparatively low incidence of r and high incidence of R_1 and R_0.

TABLE VI

Estimated frequencies of the Rh chromosomes in Manitoba whites in comparison with data from England

"Racial origin"	Number in sample	Frequency, %						
		R_1	r	R_2	R_0	R′	R″	R_z
English	792	41.72	41.13	12.78	1.95	1.21	1.06	0.15
Scottish	502	41.38	40.17	15.29	1.46	0.49	0.74	0.47
Irish	316	43.54	42.10	11.77	1.11	1.11	0.37	0.00
German	154	42.56	41.09	11.71	0.78	2.31	1.55	0.00
Dutch	113	45.11	38.79	13.88	2.22	0.00	0.00	0.00
French	263	44.30	37.00	14.67	1.51	1.01	1.51	0.00
Ukrainian	350	40.50	43.09	11.87	1.94	2.26	0.00	0.33
Polish	118	50.12	36.82	10.79	1.13	1.13	0.00	0.00
Hebrew	140	52.98	28.03	10.13	5.78	2.44	0.00	0.64
Others	351	45.64	35.00	13.94	3.12	1.20	0.80	0.30
Total	**3100**	43.48	39.55	12.87	1.91	1.24	0.73	0.22
Data from England*	**2000**	42.04795	38.86134	14.10870	2.56677	0.98349	1.18819	0.24356

* *R. R. Race, A. E. Mourant, Sylvia D. Lawlor, and Ruth Sanger. The Rh Chromosome Frequencies in England. Blood, J. Hemat. 3 : 689-695. 1948.*

Tests of Homogeneity

The homogeneity of different racial groups with respect to frequencies of O, A, and B genes may be tested by comparing two groups at a time using the Test of Homogeneity as described by Fisher (4). The same method can, of course, be applied in the case of Rh chromosomes. In both cases it is considered best to deal with the actual frequencies of phenotypes rather than the estimated frequencies of genes.

Such comparisons have been made, and typical analyses are shown in Tables VII and VIII. For some comparisons it was necessary, because of small numbers, to pool the data for the less frequent Rh phenotypes R_z, R′r, R′, R″, R_1R'', and R_0 and we have, therefore, carried this out in all comparisons, using the six phenotypic classes shown in Table VIII. The tests as we have carried them out cannot yield any information regarding homogeneity with respect to the less frequent Rh phenotypes mentioned above, but only with respect to the major phenotypes.

TABLE VII

TEST OF HOMOGENEITY OF ENGLISH AND SCOTTISH GROUPS WITH RESPECT TO ABO PHENOTYPES

Phenotype	English	Scottish	Total
O	372	244	616
A	331	188	519
B	71	55	126
AB	18	15	33
Total	792	502	1294

$\chi^2 = 3.485$. $n = 3$. $P = .95$ *approx.*

TABLE VIII

TEST OF HOMOGENEITY OF ENGLISH AND SCOTTISH GROUPS WITH RESPECT TO Rh PHENOTYPES

Phenotype	English	Scottish	Total
r	134	81	215
R_1	140	86	226
R_1r	265	171	436
R_2	117	85	202
R_1R_2	107	66	173
Others	29	13	42
Total	792	502	1294

$\chi^2 = 2.235$. $n = 5$. $P = .80$ *approx.*

The χ^2 values for tests of homogeneity of pairs of racial groups with respect to the proportion of individuals in the four phenotypes, A, B, O, and AB are shown in Table IX. The Hebrew, Ukrainian, and Polish groups do not differ significantly among themselves but they differ significantly from all other groups in the relative proportions of the four phenotypes, apart from the Hebrew vs. Irish comparison, which gives a χ^2 value close to that for the 5% level of significance. The French, German, Dutch, Irish, Scotch, and English groups do not differ statistically among themselves.

TABLE IX

χ^2 VALUES FOR TESTS OF HOMOGENEITY OF PAIRS OF RACIAL GROUPS WITH RESPECT TO THE PROPORTION OF INDIVIDUALS IN THE FOUR PHENOTYPES A, B, O, AND AB*

	English	Scottish	Irish	Dutch	German	French	Polish	Ukrainian	Hebrew
Hebrew	14.10	10.22	6.26	9.71	8.51	11.17	2.13	2.84	—
Ukrainian	60.49	39.18	23.57	20.64	22.28	34.34	2.86	—	
Polish	24.73	15.43	11.82	10.84	10.12	16.52	—		
French	1.99	5.72	5.98	1.66	1.25	—			
German	2.73	2.40	2.57	1.50	—				
Dutch	5.07	5.39	5.32	—					
Irish	4.82	0.65	—						
Scottish	3.48	—							
English	—								

* *The χ^2 value when $n = 3$ and $P = .05$ is 7.815.*

Table X gives the χ^2 values for tests of homogeneity of pairs of racial groups with respect to the proportion of individuals in six classes based on Rh phenotypes. Here the Hebrew racial group differed significantly from all other

TABLE X

χ^2 VALUES FOR TESTS OF HOMOGENEITY OF PAIRS OF RACIAL GROUPS WITH RESPECT TO THE PROPORTION OF INDIVIDUALS IN SIX Rh PHENOTYPIC CLASSES*

	English	Scottish	Irish	Dutch	German	French	Polish	Ukrainian	Hebrew
Hebrew	25.54	28.83	23.94	10.90	18.59	15.51	11.28	26.55	—
Ukrainian	3.84	5.42	3.45	1.71	4.44	12.13	12.47		
Polish	7.56	7.45	5.66	7.88	3.27	2.25	—		
French	6.01	6.73	6.77	2.90	1.28	—			
German	5.21	3.48	4.08	3.81	—				
Dutch	2.77	2.95	1.65	—					
Irish	2.03	1.56	—						
Scottish	2.24	—							
English	—								

* *The classes used were r, R_1, R_1r, R_2, R_1R_2, and a class including the remaining phenotypes, namely R_z, $R'r$, R', R'', R_1R'', and R_0.*
The χ^2 value when $n = 5$ and $P = .05$ is 11.070.

groups except the Dutch, but in this comparison the χ^2 value is close to the 5% point of significance. The remaining eight national groups furnish 28 comparisons and only two of these (Ukrainian vs. French, and Ukrainian vs. Polish) show significant differences. It should be kept in mind that even if no real differences existed in these 28 comparisons a χ^2 value large enough to indicate significance would be expected to occur by chance once in 20 comparisons. It is possible that the two largest χ^2 values in the 28 referred to above are such chance values. Such consistently large χ^2 values as occurred for the comparisons of Hebrew with other groups, however, are so unlikely to have occurred by chance that it is safe to conclude that there is a real difference between the Hebrew and other white groups in frequencies of Rh phenotypes. It follows, of course, that there is also a difference in frequencies of Rh chromosomes.

It is possible, and indeed probable, that in some of the comparisons of national groups where differences with respect to frequencies of ABO or Rh phenotypes were not statistically significant, real differences actually existed, and that more extensive data will be needed to demonstrate such differences. A nonsignificant result in such comparisons as ours indicates that either no biologic difference existed with respect to the genes studied or else that the differences were not great enough to be demonstrable with the data at hand.

Finally, an interesting test of homogeneity is shown in Table XI in which our sample of 792 individuals of English origin in Manitoba is compared with the sample of 2000 individuals in England with respect to Rh phenotypes.

TABLE XI

TEST OF HOMOGENEITY OF 2000 INDIVIDUALS IN ENGLAND AND 792 INDIVIDUALS OF ENGLISH DESCENT IN MANITOBA WITH RESPECT TO Rh PHENOTYPES

Reactions to antisera C, D, E, c	Phenotype	English (Race *et al.*) (a)	English in Manitoba (a')	Total ($a + a'$)	$\frac{(an' - a'n)^2}{a + a'}$
++−−	R_1	373	140	513	463,261
++−+	R_1r	689	265	954	257,980
++++	R_1R_2	270	107	377	68
−+++	R_2	279	117	396	428,871
−−−+	r	307	134	441	1,400,954
+−−+	R′r	16	6	22	20,527
−−++	R″	19	7	26	42,242
−+−+	R_0	42	13	55	959,376
+++−	R_z	5	1	6	640,267
+−−−	R′	0	2	2	8,000,000
		2000 (n)	792 (n')	2792	12,213,546

$\chi^2 = \frac{12,213,546}{2000 \times 792} = 7.711. \quad n = 9. \quad P = .5.$

The *P* value of 0.5 indicates that the two series do not differ significantly in distribution of Rh phenotypes. If we pool the data for R_z and R′ to avoid the zero frequency in R′ the *P* value becomes 0.95 with eight degrees of freedom, and the same conclusion is reached. The homogeneity is noteworthy in view of the data having been collected in different countries and the Rh reactions determined by different methods.

Acknowledgment

We wish again to record our debt to Dr. L. K. Diamond for supplying us with two of the sera and also for a grant to one of us (A.H.). We are indebted to the Associate Committee on Medical Research of the National Research Council, Ottawa, for a grant covering the remaining expenses of this investigation.

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ON ERGOTHIONEINE: ITS PREPARATION FROM BLOOD AND ERGOT[1]

By G. Hunter, G. D. Molnar, and N. J. Wight

Abstract

Ergothioneine has been prepared from pig blood and from ergot of rye. A poorer yield of ergothioneine was obtained from pig blood received in the Edmonton area than previously by Hunter and Eagles in the Toronto area. By a simplified method a yield of 0.26% has been obtained from ergot.

Introduction

The recent observations of Lawson and Rimington (8) on the action of ergothioneine on the thyroid gland of the rat has revived interest in this substance discovered in ergot of rye by Tanret (10) in 1909. In 1925 it was isolated from pig blood by Hunter and Eagles (5, 6) and later by Benedict, Newton, and Behre (1). The product from blood was identified as ergothioneine by Eagles and Johnson (2). It appeared probable that a supply of ergothioneine would be required for physiological and clinical studies, and this paper concerns significant observations on its preparation from pig blood and from ergot of rye.

Experimental

Preparation from Pig Blood

The methods used were essentially those already reported by Hunter and Eagles (5, 6), but our experience proved less fortunate. A routine yield obtained from about 3-gal. lots of pig blood in Toronto was about 0.7 gm. The best yield obtained in Edmonton from 10 lots of about 3 gal. of blood was 0.39 gm. and from the entire 30 gal. of blood used less than 2 gm. ergothioneine was obtained.

Preparation from Ergot

After some preliminary testing the following method was adopted.

Five hundred gm. of ergot corns was ground finely in a coffee mill. This was suspended in 2 liters water with the addition of 1 ml. glacial acetic acid. The mixture was brought to boil, with stirring at frequent intervals. While hot it was poured through cheesecloth and the residue was extracted in a filter press. The residue was washed twice, by bringing to boil with 1 liter water on each occasion, filtering, and expressing as above. The volume of filtrate and washings was 2660 ml.

The filtrate was now treated with a saturated aqueous solution of uranium acetate in very slight excess as tested, on a tile, by a 5% aqueous solution of

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[1] *Manuscript received February 7, 1949.*

Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., with Grant-in-Aid, M.P. 189a, from Division of Medical Research, National Research Council, Canada.

potassium ferrocyanide. The requisite amount of uranium acetate solution for the whole filtrate was determined on a 10 ml. aliquot. This was found to be 532 ml. It was added to the filtrate, and the mixture was well shaken. It was left to settle overnight, then the clear yellow supernatant liquid was siphoned off. The remainder was transferred to 250-ml. centrifuge bottles and further supernatant fluid collected.

The bulky precipitate in each bottle was thoroughly suspended in its own volume of water, recentrifuged, and the supernatant washings were added to the main filtrate. The washing was repeated twice. Total volume of filtrate and washings was 4450 ml.

The solution was now made 0.5 *N* acid with sulphuric acid and heated to 60° C. An aqueous suspension of cuprous oxide was added and the solution vigorously shaken. The cuprous oxide was added in slight excess as judged by a reddish tinge in the precipitate after shaking and maintaining at 60° C. for at least 30 min. The precipitate was allowed to settle. The supernatant liquid was siphoned off, and the remainder centrifuged. The copper precipitate was washed at 60° C. with its own volume of 0.5 *N* sulphuric acid, which had been brought to boil with a little cuprous oxide added. The washing was repeated twice.

The washed copper precipitate, containing some excess cuprous oxide, was suspended in hot water and freed from copper with hydrogen sulphide.

The filtrate and washings were freed from hydrogen sulphide, then brought to neutral to litmus paper by addition of warm barium hydroxide solution. At this point the solution was free from Ba and SO_4 ions.

The almost water clear filtrate was reduced *in vacuo* to about 15 ml. It was then boiled with a little charcoal, and the filtrate transferred to a boiling tube. The volume was reduced *in vacuo* to about 5 ml., when massive crystallization set in. The vacuum was released, and the contents of the tube brought into solution at near boiling point over a flame. Ten to 15 ml. absolute ethanol was added with shaking and a copious crop of crystals appeared. The whole was placed in a refrigerator for some hours and the crystals were then collected in a small Hirsch funnel, washed with absolute ethanol, and allowed to dry in the air.

A yield of 1.30 gm. of practically pure anhydrous ergothioneine was obtained. This is equivalent to 0.26% ergothioneine in the ergot used.

Recrystallization

The product from each batch of blood or ergot was kept separate for assay by colorimetric determination. A specimen of proved purity prepared by Hunter and Eagles (6) served as standard. One of the products gave no diazo test. It proved to be hypoxanthine chloride. The ergot products had small amounts of hypoxanthine present.

The crude products were suspended in water and the insoluble hypoxanthine filtered off. The filtrates were boiled with a little charcoal and recrystallization carried out as described under the preparation from ergot.

The appearance of the crystals is usually that already pictured by Hunter and Eagles, and described by Eagles and Johnson (2). Rarely more complete crystal forms are obtained.

The material is practically anhydrous when allowed to dry on the laboratory bench. For example 99.2 mgm. of air-dried material, after being heated for 1.5 hr. at 110° lost only 0.3 mgm.

The twice recrystallized product melts rather definitely with expansion and decomposition at 280° C. (uncorrected).

No further analysis of our material was deemed necessary.

Discussion

Our failure to obtain good yields of ergothioneine from pig bloods in Edmonton appears to be due to lower ergothioneine content than in pig bloods reported by Hunter (4) in 1928 in Toronto. The mean ergothioneine value for 12 Toronto pigs was found to be about 26 mgm. per 100 ml. corpuscles. Ten pig bloods tested in Edmonton in August 1948, showed a mean value of about 6 mgm. per 100 ml. whole blood, or less than half of the Toronto value. It should be noted however that the colorimetric determinations in both cases are open to question.

The evidence of Eagles and Vars (3) that diet affects the blood ergothioneine level may be the explanation for our present experience, but an adequate answer awaits further studies based on an accurate method for determining ergothioneine in blood.

In the preparation of ergothioneine from blood Hunter and Eagles (unpublished observation) occasionally observed traces of hypoxanthine in their products. In our experience hypoxanthine has been more abundant than ergothioneine in some of our crude products. When preparing ergothioneine, free base, it is easy to get rid of hypoxanthine as the free purine is very insoluble in water and readily filtered from neutral solutions of ergothioneine. But if an acid ion is present, as in the case of the preparation of ergothioneine hydrochloride, not only does the purine become soluble but it forms crystals similar to those of ergothioneine, even to showing the bright birefringence colors, under crossed Nicols, that are rather characteristic for ergothioneine and its salts. The use of cuprous oxide, as has been experienced by Pirie (9) and Lawson and Rimington (8), does not overcome the difficulty. It is not surprising that cuprous oxide precipitates purines, as its chemical action is likely to be similar to that of the copper sulphate – sodium bisulphite used in the classical method of Krüger and Schmid (7) for purine determinations.

There appears to be little or no purine present in ergot, and nitroprusside reacting SH – groups are, on our own observations, also absent, so that cuprous oxide is an eminently suitable precipitant for ergothioneine from this source. Our yields from ergot reasonably approach the maximum present in our material.

The use of cuprous oxide is not unattended with some uncertainties. We first applied it to recover ergothioneine from some residues. After the addition of some cuprous oxide suspension the whole solution set to quite a firm gel even at 60° C. The addition of more cuprous oxide liquified the gel and finally a nearly granular precipitate was obtained. On washing with cold 0.5 *N* sulphuric acid, gelatinous material again appeared, and it was proved that an appreciable amount of material was dissolved. A suitable wash solution was found in 0.5 *N* sulphuric acid with a little copper present. The solution was prepared by adding a little cuprous oxide to 0.5 *N* sulphuric acid and bringing to boil for a few minutes. The precipitate was washed with the hot acid. Thorough washing at this stage is essential to remove all ions other than SO_4—subsequently removed by barium hydroxide solution.

Acknowledgments

We wish to thank Mr. V. R. Jones in charge of Swift Canadian Co. Laboratories here for supplying us with blood. Dr. G. B. Sanford of the Dominion Laboratory of Plant Pathology here kindly supplied us with several pounds of ergot.

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THE DETERMINATION OF ERGOTHIONEINE IN SIMPLE SOLUTION AND IN BLOOD[1]

BY G. HUNTER

Abstract

The diazo method of Hunter for the determination of ergothioneine in simple solution has been adapted to the Evelyn photoelectric colorimeter. The application of the method to the determination of ergothioneine in blood is described. Suitable blood filtrates are prepared by deproteinization with standard acetic acid oxalate solutions followed by removal of reduced glutathione with Goulard's lead acetate solution, and removal of excess lead with phosphate. By the method precise recovery is obtained of ergothioneine added to human blood.

Introduction

The method described by Hunter (9) for the determination of ergothioneine was regarded as satisfactory for simple solutions, but as of a preliminary nature when applied to blood from various animals. Hunter used reputed tungstic acid filtrates for the determination.

The need for an improved method has recently been emphasized by a number of investigators, as Lawson and Rimington (12) and Astwood and Stanley (1). It was thus decided to restudy the whole problem.

In the interval since the method was first described the advent of the photoelectric colorimeter has necessitated adapting the test in simple solutions to the much more precise modern instruments. In the process of doing this it was found that altering the conditions of the original test in some details considerably increased the sensitivity of the test. Details are given in Part I of this paper for the performance of the test, along with the ergothioneine calibration curve determined for use with the macro attachment of the Evelyn colorimeter.

The problem of elaborating a satisfactory method for blood proved much simpler than might have been anticipated. Part II of this paper describes a method for the determination of ergothioneine in blood, with evidence of its accuracy.

Part I. Determination of Ergothioneine in Simple Solution

EXPERIMENTAL

Reagents

1. The *diazo reagent*, according to Koessler and Hanke (11), is made as follows. In a 50 ml. volumetric flask, immersed in a beaker with water and ice, is placed 1.5 ml. of a solution containing 9 gm. sulphanilic acid and 90 ml. of 37% hydrochloric acid per liter, followed by 1.5 ml. of 5% sodium nitrite

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[1] *Manuscript received February 7, 1949.*
Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta.

solution. The mixture is allowed to stand for five minutes, then a further 6 ml. of the nitrite solution is added. At the end of a further five minutes ice cold water is added to the mark and the contents are mixed.

2. *Alkaline buffer.* One gm. anhydrous sodium carbonate and 10 gm. anhydrous sodium acetate are dissolved in water and the volume made up to 100 ml.

3. *Ten N sodium hydroxide,* which should be crystal clear and colorless.

Method

A 1 ml. delivery pipette is inserted in the flask carrying the diazo reagent kept immersed in a 1 liter beaker of ice water. A properly matched Evelyn colorimeter tube is also put into the ice water.

If x ml. is the volume of the test solution to be used $2-x$ ml. water is added to the tube, followed by 1 ml. diazo reagent and 2 ml. alkaline buffer. Without delay, x ml. test solution is added and the solutions thoroughly mixed. In the presence of ergothioneine a clear lemon-yellow color rapidly develops. Forty-five seconds are allowed for coupling. The tube is removed from the beaker and 5 ml. 10 *N* sodium hydroxide is rapidly run in. The contents, of the constant volume of 10 ml., are thoroughly mixed. The tube is held in the hand to warm slightly and facilitate the escape of fine air bubbles produced on the addition of the strong base. After three to four minutes the solution is free from air bubbles and the final purplish pink color has fully developed. The tube is then read in the colorimeter.

A blank test carried out with 2 ml. of water is used to set the colorimeter at 100% transmittance. It should be noted that the blank is entirely colorless but has a greater transmittance than distilled water because of its greater refractive index. If this tube is stoppered it may serve as blank for several weeks.

The color fades very slowly decreasing perhaps 1 galvanometer unit in 15 to 30 min.

CONSTRUCTION OF CALIBRATION CURVE

It was first necessary to choose a suitable filter for the color developed. An absorption curve of the colored solution produced as above was determined in a Coleman spectrophotometer. The peak of absorption was found at a wave length close to 510 mμ. The Evelyn filter No. 520, transmitting in the range 495 to 550 mμ., was thus judged to be suitable. The galvanometer zero was checked.

A solution of ergothioneine containing 0.05 mgm. per ml. was used for the measurements.

The calibration curve is shown in Fig. 1. It is seen to be linear. Its mgm./density slope is 0.045. The concentration of ergothioneine is simply obtainable from the product of slope and density.*

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* *The usual calibration constant K_1 in the expression $C = L/K_1$, where C mgm. per ml. is the concentration of the chromogen in the test solution, and L is the photometric density, is 1/10th the reciprocal of the slope, i.e. 1/0.0045 or 222.*

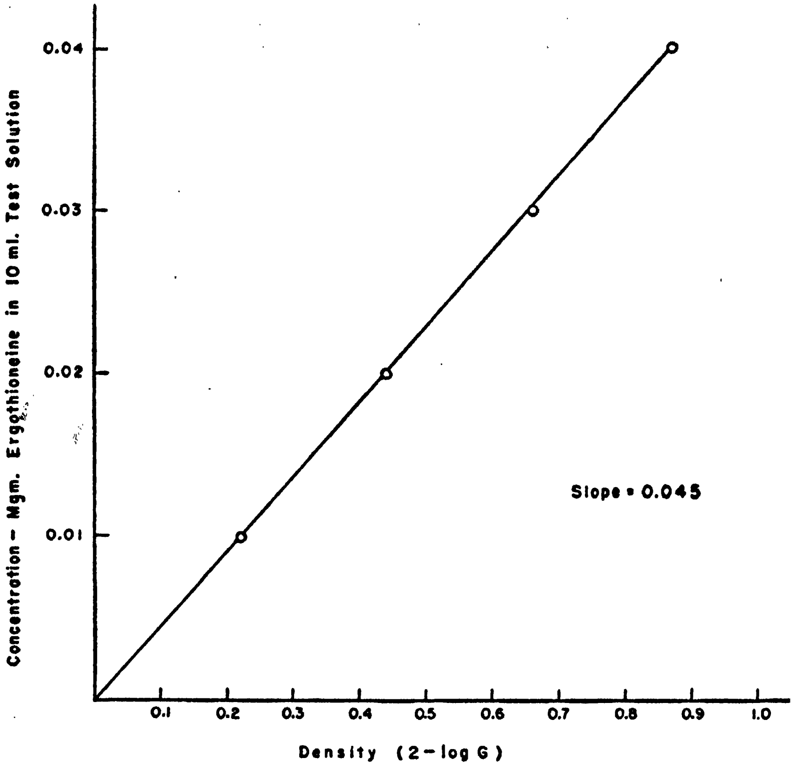

FIG. 1. *Calibration curve for ergothioneine determination.*

Part II. Determination of Ergothioneine in Blood

DISCOVERY OF MAIN FACTORS RELATING TO THE PROBLEM

In resuming the study of the determination of ergothioneine in blood it was unexpectedly found that tungstic acid filtrates rarely gave what could be described as a positive diazo test for ergothioneine. Whether in the course of 20 years the composition of commercial sodium tungstate had changed did not seem at this stage a promising approach so it was decided to remove the proteins from blood simply by heating with a suitable amount of acid, as we found most useful in the preparation procedure.

By treating such filtrates with uranium acetate and removing excess of the latter with sodium dihydrogen phosphate, it was possible to get water clear filtrates giving markedly red colors in the test. But it was rarely that the readings from such filtrates proved to be proportional. Usually, for example, a greater ergothioneine value was calculable when 1 ml. of a 1 : 10 filtrate was used, than when 2 ml. was used. This suggested a substance(s) in the filtrate inhibiting color production, less of it being present in 1 ml. than in 2 ml. of filtrate.

From a stock solution, 0.036 mgm. ergothioneine was placed in each of five test tubes. To four of the tubes was added respectively 2, 1, 0.5, and 0.2 ml.

of a blood filtrate. The volumes in all cases were made to 4 ml. with water. From each tube a 2 ml. portion was taken for the diazo test, and the galvanometer readings were recorded. Table I shows their corresponding density values.

TABLE I

DENSITY VALUES FROM ERGOTHIONEINE SOLUTIONS, WITHOUT AND WITH THE ADDITION OF DIFFERENT AMOUNTS OF BLOOD FILTRATE. SEE TEXT FOR DESCRIPTION

Solutions	Density values
Ergothioneine solution	0.398
" " + 2 ml. filtrate	0.257 (poor color)
" " + 1 ml. filtrate	0.284 (poor color)
" " + 0.5 ml. filtrate	0.482 (good color)
" " + 0.2 ml. filtrate	0.444 (good color)

The inhibiting action of 2 and 1 ml. filtrate is clear. But when 0.5 ml. filtrate is used, inhibiting action is at least largely removed, and 0.5 ml. filtrate has contributed (from difference in densities) about 0.004 mgm. to the 0.018 mgm. ergothioneine added. One might thus calculate that this blood has about 16.0 mgm. per 100 ml., whereas on a density value of 0.167, observed on 2 ml. original filtrate, the value is 3.75 mgm. per 100 ml.

This, however, is not a satisfactory solution. Having established the presence of an interfering substance it was desirable to know what it was and, if possible, to find means to remove it.

Reduced glutathione was considered a likely interfering substance. Cysteine was first tested. A few crystals added to the buffered diazo reagent before the addition of ergothioneine completely inhibited coupling. A minute amount of cysteine added permitted some coupling but little red color was produced on the addition of sodium hydroxide. This was very characteristic of the behavior of many blood filtrates treated only with uranium acetate.

On the basis of such observations the blood filtrates were precipitated with a suitable amount of the basic lead acetate preparation known as Goulard's extract (see Part II, Reagents) previously employed by Hunter and Eagles (10) in the preparation of ergothioneine and glutathione from blood. The slight excess of lead was removed from such filtrates by the addition of a suitable amount of a solution of sodium dihydrogen phosphate.

Filtrates thus prepared are suitable for the determination of ergothioneine in all bloods tested, and ergothioneine added to such bloods is quantitatively recoverable. As indicated below this claim is not made for ox blood.

To make the method suitable for routine purposes it was, however, necessary to define more accurately conditions at different points in the procedure. These concern the following main points.

(a) *Deproteinization of Blood*

That blood can be roughly deproteinized by heating with acid is recognized by all, but the method never seems to have been seriously considered in the field of blood analysis. Were it practicable it has the obvious advantages of yielding a maximum amount of filtrate to which has been added only one simple acid ion. On the face of it, of course, it would not appear to be workable as a routine method. Whole bloods differ in composition in a number of directions: hemoglobin content, plasma protein content, and in their proportion of corpuscles to plasma. The isoelectric points of serum proteins are also distinctly lower than the isoelectric point of hemoglobin.

Despite such considerations the deproteinization of blood by simply heating with acetic acid has been found suitable for our present purposes. An exhaustive study of the method has not yet been undertaken but precise enough conditions have been determined for the satisfactory removal of proteins from whole blood, corpuscles, and plasma, in dilutions in each case of either 1 : 10 or 1 : 5. The detailed volumes and standard acid solutions are given below under Part II.

Only in quite exceptional cases do such solutions yield filtrates with more than a trace of color or cloudiness.

(b) *Precipitation with Lead Acetate Solution*

It was sometimes found that when the Goulard's extract was added to the filtrate obtained by heat and acetic acid, that a very small precipitate was obtained and the supernatant liquid often remained cloudy. Under such circumstances the filtrates are not usually suitable for colorimetry as the glutathione has not been adequately removed. It was found that with whipped blood and with serum filtrates the lead precipitate did not coagulate satisfactorily. When oxalate was added to such whipped blood or serum, the subsequent lead precipitation was satisfactory. It was thus concluded that the precipitation of small amounts of glutathione by lead is an entrainment process. Hence, to unoxalated blood, oxalate should be added in the usual amount of 1.5 to 2.0 mgm. per ml. To render the lead precipitation more uniform and certain for routine oxalated bloods, it was decided to incorporate oxalate in the standard acetic acid solutions used for protein precipitation. (See Part II, Reagents.)

(c) *Elimination of Uranium Acetate Precipitation*

Having achieved satisfactory conditions for the removal of protein and the removal of glutathione it appeared that precipitation with uranium acetate might be dispensed with. This was finally decided on in the light of the observation that its use leads to slight loss of ergothioneine, as shown in the following experiment.

Four lots of 0.5 ml. blood (human) were deproteinized. The filtrate from one of these, (*a*), was precipitated directly with lead acetate, but the other three filtrates were precipitated first with the following amounts of a saturated aqueous solution of uranium acetate:—(*b*), 0.02 ml.; (*c*), 0.04 ml.; and (*d*), 0.06 ml. The ergothioneine found, mgm. per 100 ml., was in each case:—(*a*), 7.4; (*b*), 7.0; (*c*), 6.7; and (*d*), 6.4.

Whether this loss is due to the direct precipitation of the ergothioneine with the uranium acetate, or to its entanglement in the subsequent lead precipitate has not been determined.

EXPERIMENTAL

Reagents

(*a*) *Acetic Acid – Oxalate Solutions*

The solutions as indicated in Table II are used.

TABLE II

ACETIC ACID – OXALATE SOLUTIONS FOR DEPROTEINIZATION OF WHOLE BLOOD, CORPUSCLES, AND PLASMA IN 1 : 10 AND 1 : 5 DILUTIONS

For *1 in 10* dilution	
Whole blood	1 vol. + 9 vol. 0.0045 *N* acetic acid
Corpuscles	1 vol. + 9 vol. 0.0030 *N* acetic acid
Plasma	1 vol. + 9 vol. 0.0055 *N* acetic acid
The above acetic acid solutions are made to contain 160 mgm. sodium oxalate per liter.	
For *1 in 5* dilution	
Whole blood	1 vol. + 4 vol. 0.0100 *N* acetic acid
Corpuscles	1 vol. + 4 vol. 0.0070 *N* acetic acid
Plasma	1 vol. + 4 vol. 0.0125 *N* acetic acid
The above acetic acid solutions are made to contain 360 mgm. sodium oxalate per liter.	

Of course it is only for special purposes that the plasma solutions are required, as ergothioneine has not been shown to be present in plasma.

(*b*) *Goulard's Extract* (8)

Place 220 gm. lead acetate [$Pb(CH_3COO)_2 . 3H_2O$] and 140 gm. litharge or lead monoxide [PbO] in a 2 liter Erlenmeyer flask with about 1 liter distilled water and bring to boil, with stirring. Maintain at boil for 30 min. Cool and filter through a gravity funnel into a 1 liter volumetric flask. Wash filter with carbon dioxide free water and make to mark. The reagent should be kept stoppered.

(*c*) *Phosphate Solution*

Ten gm. sodium dihydrogen phosphate [$NaH_2PO_4 . H_2O$] to 100 ml. with water.

Method

To 0.5 ml. whole blood in a centrifuge tube add 4.5 ml. 0.0045 *N* acetic acid – oxalate solution. Mix with a glass rod. Immerse in a faintly boiling water bath and stir. Coagulation takes place in one to two minutes. By rubbing the glass rod on the side of the tube the gas bubbles are detached from the coagulated protein. Centrifuge. Decant the warm supernatant liquid into another tube and drain well.

Add to the supernatant liquid one drop (0.05 ml.) of Goulard's extract. Mix and tilt the tube to catch any drops on the sides. Centrifuge. Decant as before into another tube, and add one drop phosphate solution. Mix as before and centrifuge. Decant into another tube. This is the test solution – about 3.7 ml. volume.

The test is now carried out with the diazo reagent and buffer as described in Part I for the determination in aqueous solution. At least two aliquots should be taken – usually 2 and 1 ml. – to test proportionality of readings. The amount of ergothioneine in the test solution is obtained as already described.

Recovery of Added Ergothioneine

Ergothioneine added to the original blood is quantitively recoverable by the method, as shown in Table III.

TABLE III

RECOVERY OF ERGOTHIONEINE ADDED TO HUMAN BLOODS

Subject	Mgm. ergothioneine per 100 ml.			
	Original blood	Added	Found	Calculated
M	4.15	2.85	7.25	7.00
T	(*a*) 1.25 (*b*) 1.25	2.30 4.60	3.50 6.00	3.55 5.85
S	(*a*) 4.35 (*b*) 4.35	4.25 8.50	8.60 12.90	8.60 12.85

The recovery of ergothioneine, added to the original blood so that it goes through the whole extraction process in the preparation of the final filtrate for the test is perhaps the best criterion of the method. Subject T was a case of hyperthyroidism and shows the lowest ergothioneine value we have so far found in blood. For the reason that the original ergothioneine content was very low this blood presents a rather rigorous criterion for the recovery of small amounts of ergothioneine added to it.

Distribution of Ergothioneine in Blood

Table IV serves as a protocol of the colorimetric assay method and as a further check of its accuracy. The concordance of values found for whole blood with those calculated from corpuscles and plasma values would indeed have been better had the plasma also been precipitated at a 1 : 10 dilution.

TABLE IV

PROTOCOL FOR TWO HUMAN BLOODS

Subject	Corpuscles, vol. %	Analysis material	Filtrate dilution	Aliquot for test, ml.	Photometric density	Ergothioneine in 0. 1 ml. blood, mgm.	Ergothioneine mgm./100 ml.	
							Found	Calculated*
K	48.05	Whole blood	1 : 10	2	0.235			
				1	0.121	0.0054	5.4	5.5
		Corpuscles	1 : 10	2	0.475			
				1	0.233	0.0105	10.5	
		Plasma	1 : 5.5	2	0.069			
				1	0.036	0.0009	0.9**	
S	33.33	Whole blood	1 : 10	2	0.258			
				1	0.126	0.0057	5.7	6.1
		Corpuscles	1 : 10	2	0.688			
				1	0.347	0.0156	15.6	
		Plasma	1 : 5.5	2	0.109			
				1	0.055	0.0013	1.3**	

* *From content of corpuscles and plasma and volume % corpuscles.*
** *Not to be interpreted as ergothioneine, see text.*

It was a surprise to find that a significant yellow color is always found in plasmas even with a minimum of corpuscles or their constituents present. Expressed as ergothioneine this color is usually equivalent to 0.5 to 1.0 mgm. per 100 ml. plasma. There is no reason to believe that any of this color is due to ergothioneine. It is most likely due to histidine and (or) tyrosine. If we assume that 1 mgm. of each of these amino acids is circulating in 100 ml. plasma then, at a 1 : 5 dilution with a 2 or even 1 ml. aliquot they come just within the range of sensitivity of the diazo reagents. However with the limitation of the coupling time to 45 sec. perhaps a negligible proportion of the color arises from histidine, and the greater amount from tyrosine, which couples faster than histidine.

The interference indeed is considered to be insignificant in the determination of ergothioneine in whole blood or corpuscles, but it is of interest in the still open question as to whether plasma has a low level of circulating ergothioneine. Values found for plasma by the present method should not be interpreted as indicative of the presence of ergothioneine.

It may be observed from Table IV that the 1 ml. aliquot of filtrate gives very nearly in all cases just half the ergothioneine value obtained from the 2 ml. aliquot. This is so, perhaps fortuitously, also in the case of the plasmas. In the occasional blood filtrate, a proportionately lower value is found with the 2 ml. aliquot in which case the color hue is not likely to be of a characteristic bright purplish pink but more yellow, indicating the presence of interfering substance (s). In most of such cases the use of a 0.5 ml. aliquot will give a color proportional to that of the 1.0 ml. aliquot in which cases the values for the 2 ml. aliquot should be discarded.

To date, upwards of 100 whole bloods have been tested with only occasional evidence of interference with color development. From several ox bloods examined there is evidence of gross interference by some constituent not yet identified. Combined uric acid, already demonstrated to be present in ox blood by Bulmer, Eagles, and Hunter (6), and earlier by Davis, Newton, and Benedict (7), may be a factor in the present problem.

The simplicity of the present method may here be emphasized. Two to four test filtrates can be prepared in 10 to 15 min., with simultaneous deproteinization and the use of a modern clinical centrifuge. It should be relatively simple to adapt the method to the micro Evelyn attachment.

Discussion

In a note on the determination of ergothioneine in blood filtrates Behre (2) in 1932 stated that "no great reliance can be placed on figures so far obtained." It is unlikely that such a statement repeated today could be contradicted.

The observation of Benedict and Newton (5) that glutathione is precipitated by silver lactate, used for the determination of uric acid in blood, would appear to discredit all ergothioneine values reported on the basis of the use of uric acid reagents. It is thus unnecessary here to discuss the various publications on the ergothioneine content of blood obtained without adequate fractionation by such unspecific reagents.

There is one point, however, of some interest in the history of this subject. It has been stated at the beginning of this paper that tungstic acid filtrates of blood generally failed to give positive diazo tests for ergothioneine whilst the values reported in 1928 by Hunter (9) were obtained from reputed tungstic acid filtrates.

Benedict and Newton (3) recommended the use of molybdic acid filtrates on evidence that ergothioneine is precipitated by tungstic acid. On their further evidence that molybdic acid precipitates some glutathione, they (4) later recommended a tungstic-molybdic acid mixture, and stated that the ergothioneine values in such filtrates are about twice those obtained in tungstic acid filtrates.

A pig blood known to contain 14.8 mgm. ergothioneine per 100 ml. was precipitated with tungstic acid at a 1 : 10 dilution in the usual way, and a portion similarly treated with molybdic acid according to Benedict and Newton (3). Diazo tests were carried out on a 2 and 1 ml. aliquot of each, and remarkably enough, good ergothioneine diazo colors were obtained from the molybdic acid filtrate but poor yellow colors on the tungstic acid filtrate. The 2 ml. aliquot of the molybdic filtrate indicated 9.0 mgm. and the 1 ml.

filtrate 10 mgm. ergothioneine per 100 ml, or about 60% of the true ergothioneine content. Significantly both filtrates showed a positive nitroprusside test indicative of reduced glutathione.

This observation would appear to solve the puzzling discrepancy between the observations of Hunter in 1928 and the present ones. It is highly probable that the commercial tungstic acid used by Hunter in 1928 was composed partly of molybdic acid.

No attempt need here be made to explain the different behaviors of tungstic and molybdic acid, as the present method is not concerned with either.

It may, however, be stated that the values already published by Hunter (9) on the ergothioneine content of bloods probably represent not more than 75% and in some cases much less of the true values for the bloods reported.

It is hoped to report further, at an early date, on the normal levels of ergothioneine in human and animal bloods.

It would appear that the method here described for the determination of ergothioneine in blood will make possible a better understanding of its physiological and perhaps clinical significance.

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NOTE ON THE ERGOTHIONEINE CONTENT OF ERGOT FROM DIFFERENT PLANT HOSTS[1]

By G. Hunter, S. G. Fushtey, and D. W. Gee

Abstract

A method is described for the determination of ergothioneine in ergot sclerotia obtained from the host plants *Agropyron*, *Bromus*, *Calamagrostis*, *Elymus*, *Hordeum*, and *Secale*.

Ergothioneine is present in all ergots examined at levels from 0.157 to 0.531%. The average concentration in ergot of barley is 0.376% and in ergot of rye 0.336%.

Introduction

As ergothioneine can not yet be obtained by synthesis and as large volumes of blood, under the most favorable conditions, must be processed to obtain the substance in amounts useful for physiological or clinical studies, it was decided to examine more closely the ergothioneine content of ergot.

The only indications of the percentage of ergothioneine in ergot are to be found in the yields obtained from the few records of its isolation available in the literature. In chronological order such are: Tanret (6) 0.10% as hydrated hydrochloride; Eagles (1) 0.065% as anhydrous free base; Pirie (5) 0.18% as anhydrous free base, and Hunter, Molnar, and Wight (4) 0.26% as anhydrous free base.

It appeared that the method of Hunter (3) might, without much difficulty, be applied to the determination of ergothioneine in ergot sclerotia collected by one of us (S.G.F.) in the course of another study. This paper describes a method for the determination of ergothioneine in ergot and the values found in the fungus picked from different host plants.

Experimental

All analyses were carried out on air-dried material and the values found expressed on the same basis.

The routine analytical procedure aimed at a sample weighing 30 to 50 mgm. of material. The weight of many single sclerotia fell in this range. Large sclerotia were halved and, when the sclerotia were very small, as from *Calamagrostis canadensis*, 20 to 30 sclerotia were required for the sample.

Method

Thirty to 50 mgm. of material is placed in a 15 ml. graduated centrifuge tube. About 4 ml. water and 1 drop of 2 *N* acetic acid are added, and the tube is placed in a boiling water bath for 10 min. The contents are then poured into a small mortar, the tube being carefully set aside for return of this material.

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[1] *Manuscript received February 7, 1949.*

Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., and the Dominion Laboratory of Plant Pathology, Edmonton, assisted by a grant to one of us (D.W.G.) from the Committee on Allocation of Medical Research Grants of the Faculty of Medicine.

The ergot is finely ground, and quantitatively returned to the tube by washing pestle and mortar with a further 5 to 6 ml. water. The tube is placed in the boiling water bath for a further 5 min. It is centrifuged. The total volume, here recorded, is taken for the dilution factor in the calculation. The supernatant liquid is carefully transferred into a plain 16 ml. test tube fitting the centrifuge and treated with a saturated solution of uranium acetate to slight excess as determined by test with a 5% solution of potassium ferrocyanide on a tile with a small drop of the supernatant fluid. From 0.1 to 0.2 ml. of the uranium solution is usually required. The tube is again centrifuged, and the supernatant fluid transferred to another 16 ml. test tube. The slight excess of uranium is removed from this solution by the addition of one drop (0.05 ml.) of 10% solution of sodium dihydrogen phosphate. The solution should be well mixed and allowed 15 to 20 min. for complete coagulation of the precipitate before centrifugation. The supernatant liquid is poured into another tube. This is the test solution.

Colorimetric Determination

Aliquots from the test solution are measured by the diazo method described by Hunter (3). At least two different aliquots should show strict linear transmittance.

The values for the ergots examined are shown in Table I.

Discussion

(*a*) *Of Method*

It may be seen that the preparation of the ergot extract suitable for colorimetric determination follows the first two steps in the isolation procedure used by Hunter, Molnar, and Wight (4). In the isolation procedure however the ergot was extracted with only about five volumes of water while here about 200 volumes of water are used. The relatively high concentration of ergothioneine in ergot permits this large dilution at the outset and makes negligible the volume occupied by the ergot residue. When the material is finely ground as described it is assumed that after the second heating the ergothioneine is evenly distributed in solid and liquid.

From this point on, small amounts of ergothioneine are of course left behind in each transfer and the approximately 10 ml. volume recorded is diluted by about 2% by the subsequent addition of the uranium and phosphate solutions, so that the values finally recorded for ergothioneine in the ergot are below the real values by probably 3 to 5%.

At the dilution used for extraction, aliquots greater than 1 ml. tend to give values less than those obtained by smaller aliquots—indicating the presence of traces of substance(s) in some test solutions interfering with color development. By the use of smaller aliquots such substances are diluted beyond their range of interference and strictly linear transmittances are nearly always easily obtainable. For this reason the method of determination was considered adequate for present purposes.

(*b*) *On Occurrence in Ergot*

Except in a few cases the analyses were carried out on single sclerotia in triplicate. It may be seen from Table I that about a 50% difference in

TABLE I

THE ERGOTHIONEINE CONTENT OF AIR-DRIED ERGOT SCLEROTIA CLASSIFIED ACCORDING TO HOST PLANT

Host	% Ergothioneine	
	In separate samples	Average
Agropyron Smithii Rydb.	0.180 0.261	0.220
Agropyron repens (L.) Beauv.	0.333 0.430 0.305	0.356
Bromus inermis Leyss	0.381 0.362 0.530	0.424
Calamagrostis canadensis (Michx.) Beauv.	0.162 0.157	0.160
Elymus innovatus Beal	0.310 0.329	0.320
Hordeum vulgare var. *trifurcatum* Schlecht	0.334 0.318 0.385	0.344
Hordeum vulgare L. (immature, 1948)	0.483 0.531 0.442	0.485
Hordeum vulgare L. (mature, 1948)	0.325 0.455 0.372	0.384
Hordeum vulgare L. (mature, 1946)	0.402 0.398	0.400
Hordeum vulgare L. (mature, 1947)	0.231 0.298	0.265
Secale cereale L.—1948—Drumheller	0.299 0.183 0.215	0.232
Secale cereale L.—1948—Edmonton (mature)	0.383 0.326 0.368	0.359
Secale cereale L.—1948—Edmonton (immature)	0.233 0.293 0.202	0.243
Secale cereale L.—1947—Edmonton (mature)	0.448 0.406 0.401	0.418
	0.364 0.474 0.445	0.428

percentage ergothioneine may be found in different samples of the same variety of ergot, though generally in our few observations the variation is much less. The five specimens of ergot of barley recorded in Table I show an over-all average of 376 mgm. ergothioneine per 100 gm. with a range from 531 to 231. In the case of the five specimens of ergot of rye the over-all average is 336 mgm. ergothioneine per 100 gm. with a range from 474 to 183. The ergots from *Bromus* and *Agropyron* contain ergothioneine in about the same concentrations as ergots from barley and rye. The lowest values were found in the ergot from *Calamagrostis* but here the sclerotia are very small (little more than 1 mgm. each) and some extraneous material may have been in the sample.

The biochemical synthesis of the thiolglyoxaline nucleus in the only two sources in which it is yet known to exist namely, the fungus ergot, and mammalian red blood corpuscles, is quite obscure. The only suggestion so far offered is to be found in the paper by Eagles and Vars (2) who give some evidence that a thiolglyoxaline nucleus is contained in zein, the main protein present in maize. The findings of Eagles and Vars have not been confirmed so the presence of the thiolglyoxaline nucleus in any protein has yet to be proved.

Assuming, for a moment, the correctness of Eagles and Var's observation that a thiolglyoxaline exists in zein, an explanation of their findings with pigs fed maize and pigs fed garbage is possible. Yet it is difficult to believe that such an explanation is adequate for the general occurrence of ergothioneine in blood corpuscles. The cereal proteins have not as yet been examined for the thiolglyoxaline nucleus. Were it found to be present in the proteins of rye, or barley or other ergot hosts perhaps there would be no problem of special interest for the plant or animal physiologist. But as matters now stand the origin of the thiolglyoxaline nucleus in red blood corpuscles and in ergot would appear to be a subject of considerable biochemical interest.

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 27, SEC. E. OCTOBER, 1949 NUMBER 5

THE EFFECT OF LIVER EXTRACT PREPARATION ON BONE MARROW EXPLANTS[1]

BY K. E. ARMBRUST AND H. D. BETT

Abstract

A study has been made of the effect of various concentrations of several liver extract preparations on the migration of cells, chiefly leucocytes, from explants of guinea pig bone marrow. The migration has been shown to be stimulated by an extract of liver, which shows incomplete activity in the treatment of pernicious anemia. Two clinically active fractions have been shown to be inactive in the stimulation of cell migration. The migration of cells from explants cannot be used under the conditions described as a test for antipernicious anemia activity of liver extract preparations.

Introduction

Many attempts have been made to develop a laboratory procedure for the determination of the potency of liver extracts intended for the treatment of pernicious anemia. Overbeek and associates (8, 9) described a method involving the migration of cells from explants of guinea pig bone marrow. When these explants were cultured in a coagulum of plasma, cells, chiefly leucocytes, migrated to form a reasonably sharply defined zone of migration. The addition of a clinically active liver extract, in a series of dilutions, was said to stimulate the migration. The effect of the liver extract on the area of migration was used as an index of the potency.

Pace and Fisher (10) followed this work and claimed that the clinical potency of the extracts could be related to the dilution of the extracts at which maximal migration of the cells from bone marrow explants occurred. A curve was constructed to show this relationship. Young and Bett (13), however, could not show this relationship, and reported that no definite relationship between bone marrow response and antipernicious anemia activity could be demonstrated with this method. Subsequent work by Armbrust and Bett (2) indicated that a relationship could be established by a modification of the method used by Pace and Fisher. At this time it was stated that the bone marrow explant test might be useful in determining whether or not a liver extract had antipernicious anemia activity.

During the past year, eight fractions of liver extract have been tested clinically and by the bone marrow test. Four of these gave positive results in both tests. Tests on the remaining four fractions have indicated that it

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[1] *Manuscript received May 7, 1949.*

Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont.

is possible to separate bone marrow migration factor from antipernicious anemia substance. These are the results that are presented in this paper.

Method

The method used for the bone marrow test was essentially as previously described (2). The preparation of the bone marrow was improved to some extent by cutting on plastic rather than wood. This made it possible to get pieces more nearly even in size and more regular in shape. Another refinement in the method was to place the dishes, from which the bone marrow was to be distributed, around the circumference of a 15 cm. Petri dish. The cover of this dish had a hole drilled through it 1 cm. from the edge and 1 cm. in diameter. The bone marrow could be reached by inserting a pipette through this hole. Since the top could be turned to each dish, it was unnecessary to remove the cover each time a piece was to be placed in a culture dish. In this way the marrow was protected from contamination. Six pieces of bone marrow were placed in each culture dish as shown in Fig. 1. In each test of a liver extract two dishes were used at each of two concentrations, namely 0.16×10^{-3} and 0.33×10^{-3} ml. of liver extract per ml. of medium. In each test two dishes, to which no liver extract had been added, were also used.

The area of migration for each piece of bone marrow was measured at 30 diameters. Figs. 2 and 3 show two pieces at a magnification of 25 diameters. The results reported in this paper are for three separate tests. The average area of migration in the case of the control and at each concentration is therefore based upon results with 36 pieces of bone marrow. The temperature of the room in which the test was conducted was about 25° C.

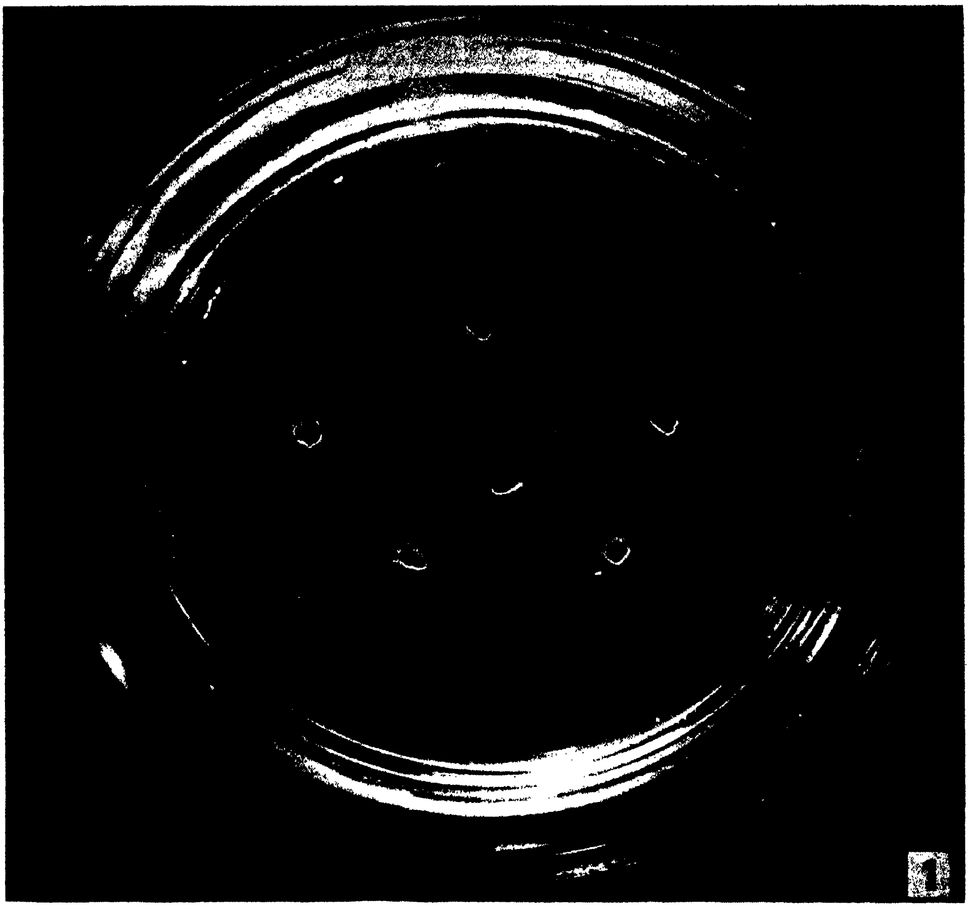
Each ml. of liver extract fraction was prepared from the same weight of liver and the clinically active solutions contained 15 units per cc. when tested on pernicious anemia patients.

Results

Table I shows the results of tests of four fractions of liver extract. The standard deviation of a single observation was 30 units. The unit refers to the smallest squared area (4 sq. mm.) on graph paper.

Fraction 137 was considered on clinical trial to be incompletely active in the treatment of pernicious anemia. Clinically active material was fractionated into two parts to produce Fractions 139 and 140. Further purification of Fraction 140 provided Fraction 143. Both 140 and 143 were shown to be clinically active extracts. In the bone marrow test, 143 showed no response to a change in concentration of the extract in the medium and 140 showed less response than 137 or 139. The results on 139, 140, and 143 were obtained in simultaneous tests. Since fraction 140 produced a maximal clinical response, it appears unlikely that any antipernicious anemia activity can be present in Fraction 139. However, this fraction has not been subjected to clinical test. These results indicate a separation of antipernicious anemia substance and migration factor.

PLATE I



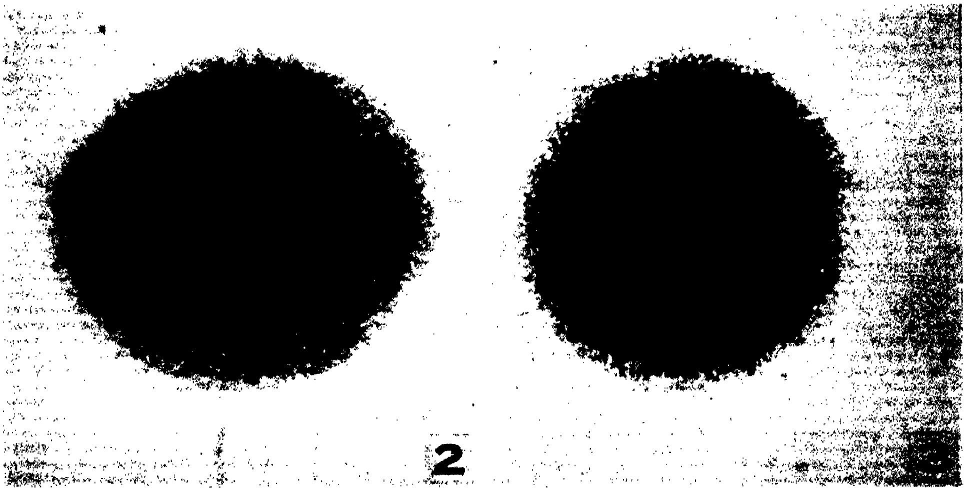


FIG. 1. *Bone marrow explants after incubation*, 2×.
FIGS. 2 AND 3. *Two explants showing area of migration as it would be seen on projection*, 25×.

TABLE I

EXTENT OF MIGRATION PER PIECE OF BONE MARROW

(The number of squares, having an area of 4 sq. mm. each, traced on graph paper)

Liver extract	Ml. of liver extract per ml. of medium		
	0.0 ml.	0.16×10^{-3} ml.	0.33×10^{-3} ml.
	Extent of migration		
Fraction 137	100 squares	125 squares	165 squares
Fraction 139	80	130	165
Fraction 140	90	100	110
Fraction 143	65	70	60

During the course of this investigation, tests were carried out in which the migration, at the various concentrations of liver extract, was very great. Tests on the serum indicated the presence of a factor that stimulated the migration. At normal concentration of serum in the medium, the average migration was 120 units; at one-half concentration, 108 units; and at one-quarter concentration, 83 units.

It has not been unusual to have a reduction in migration when a concentration of 0.3×10^{-3} ml. of active liver extract per ml. of medium has been exceeded. This was not the case with Fraction 139. The reduction in response with cruder fractions supports the evidence of previous workers (9, 10) for peak values in areas of migration at certain concentrations. Thus it seems that factors responsible for inhibiting the migration may have been removed in the purification of Fraction 139.

Clinical Results

The clinical testing of these extracts was carried out in the Department of Medicine at the Toronto General Hospital.

Extract 137

This extract was administered to a male patient 69 years of age, with an initial erythrocyte count of 1.3 millions per cu. mm. and a haemoglobin of 31% (Sahli-Leitz Haemoglobinometer 15.6 gm. = 100%). One cubic centimeter of extract was given and 1 cc. after two weeks had elapsed. During a 30-day period the erythrocyte level did not exceed 2.0 million per cu. mm. and the haemoglobin did not rise higher than 48%. A marked reticulocytosis (33%) appeared on the sixth day after the first injection and a lesser response (19%) on the sixth day after the second injection. Subsequent treatment with an active liver extract (15 units per cc.) used routinely in the clinic, produced the usual increase in erythrocytes and a third reticulocyte response (13%) on the sixth day after injection. The active extract was given in ½ cc. doses each week for three weeks. At the end of this period the erythrocyte level had reached 3.5 million per cu. mm. and the haemoglobin 75%.

Extract 140

This extract was administered to a male patient, 51 years of age, with an initial erythrocyte count of 2.1 million per cu. mm. and a haemoglobin of 56%. One-half cubic centimeter of extract was given each week for four weeks. On the sixth day after the first injection a reticulocyte peak of 24.6% had been reached. At the end of the test period the erythrocyte count was 4.0 million per cu. mm. and the haemoglobin 80%.

Extract 143

This extract produced a striking initial haematopoietic response. Its power to maintain pernicious anemia patients is under investigation.

The extract was administered to a female patient, 53 years of age, with an initial erythrocyte count of 2.7 million per cu. mm. and a haemoglobin of 64%. One cubic centimeter of the extract was given followed by 1 cc. after two weeks had elapsed. A reticulocyte peak of 17.8% was reached on the fifth day after the initial injection. A good subjective response was reported at this time. At the end of the four week test period the erythrocyte count was 4.2 million per cu. mm. and the haemoglobin 92%.

A second patient was treated with this extract. A male patient, 53 years of age, with an initial erythrocyte count of 1.1 million per cu. mm., and a haemoglobin of 27%, received 0.5 cc. doses of this extract at intervals of one week. Five days after the first injection the reticulocytes had reached a peak of 46.4%. At the end of the first week the erythrocyte count was 2.4 million per cu. mm. and the haemoglobin 58%. At the end of four weeks the erythrocyte count was 4.0 million per cu. mm. and the haemoglobin 72%. This patient has been maintained on 1 cc. of this extract every two weeks from December 1947 to the present.

Discussion

Although it has been shown that migration of cells in bone marrow explants is not an indication of the antianemia value of liver extracts, the work has not progressed to the point where a statement can be made as to the nature of the substance responsible for the stimulation of the migration.

It would appear that the migration phenomenon is something apart from the erythropoietic response observed in patients with macrocytic anemia when treated with liver extract (7), folic acid (1, 4, 5, 6, 11), or vitamin B_{12} (11, 12). Patients treated with liver extract from which the migration factor had been removed showed the usual clinical response. From our results it is apparent that the migration is not stimulated by the specific antipernicious anemia substances contained in liver extract. If we assume that vitamin B_{12} is the antipernicious anemia factor, then it is not the migration factor. The folic acid content of the extract with which we started is low (less than 1.0 μgm. per ml.). Preliminary work has shown that this substance is required at a higher concentration than this, before any significant stimulation of the bone marrow *in vitro* takes place. Berry *et al.* (3) have shown the presence of a factor, in certain preparations of liver extract and vitamin B_1, that increased the neutrophile count in patients suffering from malnutrition. Since this appears to be a direct effect on the delivery of granulocytes from the bone marrow it may be of the same nature as the substance with which we are concerned.

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ELECTRON MICROSCOPY OF TYPHUS RICKETTSIAE[1]

By C. E. Van Rooyen and G. D. Scott

Abstract

This report contains an account of a systematic study on the morphology of five rickettsiae pathogenic to man, investigated with the electron microscope. The work has bearings on the biology, antigenic structure, and taxonomy of the rickettsiae.

Technique

We have used an RCA type E.M.U. model electron microscope. Well washed suspensions of rickettsiae were inoculated on formvar covered 200 gauge stainless steel wire mesh mounts. The latter were repeatedly washed with water, allowed to dry, and fixed with osmic acid vapor.

All preparations were examined under the microscope before and after shadowcasting.

Epidemic Typhus

A (Brienl) strain of *R. prowazeki* was cultivated in the yolk sac membrane of the embryonated egg and rickettsiae were separated by extraction with ether, followed by differential centrifugalization and washing in saline according to the method of Craigie (1).

Unshadowcast mounts revealed structures of coccobacillary shape exhibiting numerous internal masses and granules of varying electronic density. The whole appeared to be surrounded by an envelope previously described in unshadowcast pictures published by Plotz, Smadel, Anderson, and Chambers (3). Mounts were next shadowcast with chromium and measurements showed that rickettsiae varied from 0.5 μ to 2.0 μ in length, 0.3 μ to 0.7 μ in breadth, with an average size of 1.1 μ by 0.5 μ.

The latter findings (see Fig. 1) suggest the presence of an outer area of low electronic density analogous to a bacterial envelope. *R. prowazeki* is composed internally of structurally and electronically dense masses surrounded by apparently fluid substance. During the process of drying the periphery of the organism collapses leaving the central masses intact and projecting through the surface (as indicated from the contour of shadows) so as to convey the appearance of an electronically translucent periphery or envelope. The relationship of these two constituent parts of the rickettsia is somewhat analogous to that of a fluid ectoplasm and a granular endoplasm. Appearances rather similar to these have been reported in preparations of vaccinia virus treated with molar sodium chloride and shadowcast with gold by Dawson and McFarlane (2). Several elongated forms were observed showing central constriction suggestive of the beginning of binary fission.

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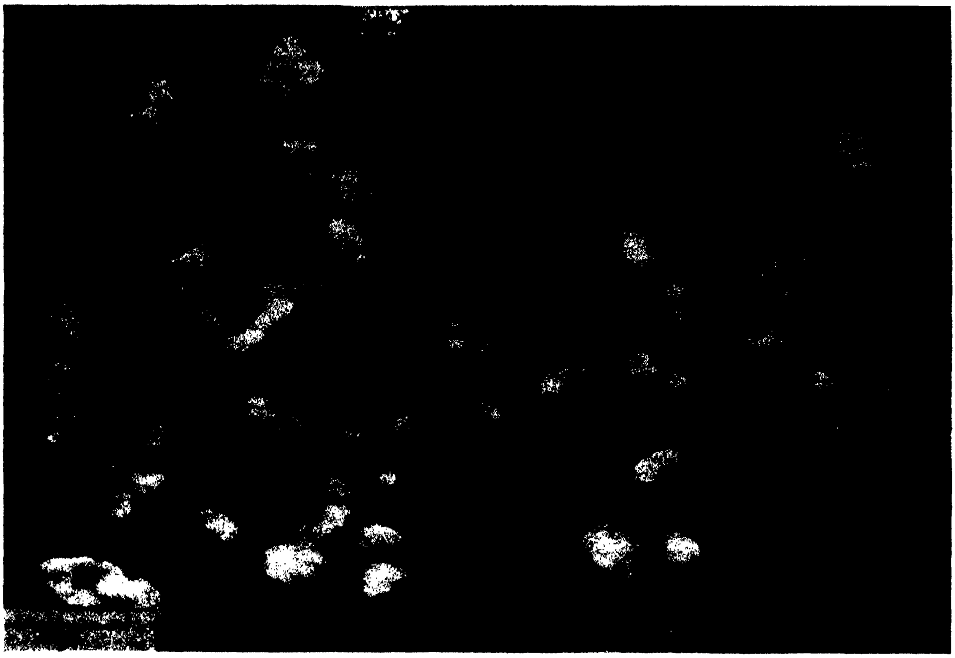
[1] *Manuscript received May 12, 1949.*

Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont. and McLennan Laboratory, Department of Physics, University of Toronto.



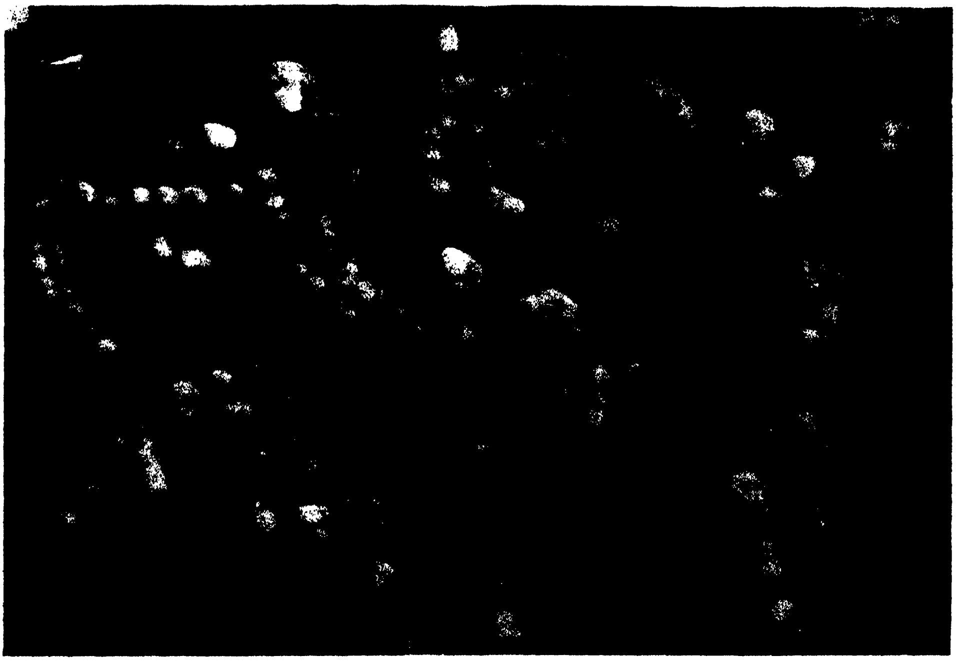
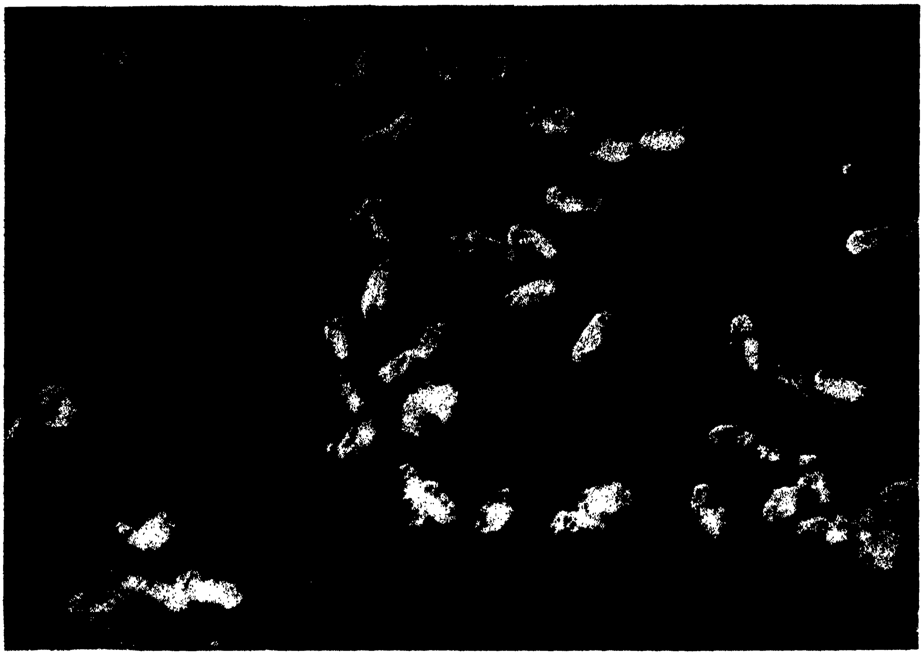


FIG. 1. *R. prowazeki.* ×20,000
FIG. 2. *R. mooseri.* ×20,000

All specimens shown have been shadowcast with chromium; the shadow to height ratio is approximately 4 : 1.



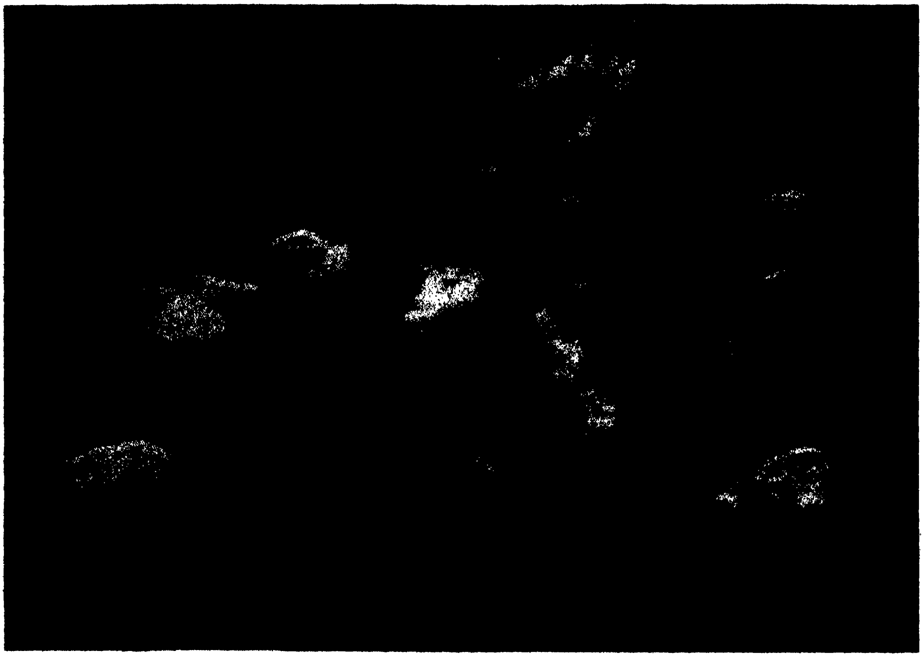


FIG. 3. *R. burneti.* ×20,000
FIG. 4. *R. burneti.* ×45,000

All specimens shown have been shadowcast with chromium; the shadow to height ratio is approximately 4 : 1.

Murine Typhus

The Wilmington strain of *R. mooseri*, grown on the yolk sac and concentrated by the manner already described, was used. Unshadowed pictures suggested the presence of more internal structure in comparison with *R. prowazeki.* In size *R. mooseri* varied from 0.7 to 1.3 μ in length, 0.35 to 0.6 μ in breadth, with an average size of 1.0 μ by 0.45 μ.

The peripheral clear area surrounding the center of the rickettsia, which we call an "envelope", appears to fit more tightly than in *R. prowazeki*; also the central masses seem to be more granular and evenly distributed throughout the organism (see Fig. 2). Individual rickettsiae showed large bipolar electronically dense masses, concentrated at each end. Evidence of binary fission was also observed.

Although *R. mooseri* (Wilmington) shows slightly less variation in size, being on the average slightly smaller than *R. prowazeki* (Brienl), we doubt whether these rickettsiae can be differentiated with certainty by electron microscopy unless the looseness of the envelope be accepted as a criterion.

Q. Fever

A concentrated suspension of *R. burneti* (Nine mile strain) was supplied by courtesy of Dr. R. L. Gauld, Army Medical School, Washington. The material was rewashed with ether and concentrated by differential centrifugalization.

Unshadowed photographs showed little detail, but after shadowing with chromium considerable structure was revealed. *R. burneti* was found to measure 0.6 μ to 0.9 μ in length, and 0.25 μ to 0.4 μ in breadth, with average size of 0.73 by 0.32 μ (see Fig. 3). Greater uniformity in size range was observed than in the case of *R. prowazeki* and *R. mooseri.* The distribution of internal structure showed considerable regularity in arrangement (see Fig. 4). The latter consisted of circular or oval shaped bodies about 120 mμ in length, 50 mμ in width. Numerous extracellular particles of about 100 mμ in diameter (see Fig. 4) were also noticed and these may have been extruded from ruptured rickettsiae. The envelope was not so noticeable and when present appeared to fit closely over the central mass.

Rickettsialpox

A strain of *R. akari*, received through courtesy of Dr. R. J. Huebner, U.S. Public Health Service, was propagated in the yolk sac of embryonated eggs and a washed suspension of rickettsiae was obtained as previously. Thereafter electron microscope mounts were prepared and pictures secured before and after shadowcasting.

R. akari showed relatively little size variation, the average being 0.9 to 1.4 μ by 0.6 μ. The envelope was evident and electronically dense masses within the organism were distributed throughout (see Fig. 5).

Rocky Mountain Spotted Fever

Photographs of *R. rickettsi* were also obtained. Antigen prepared from the "R" strain isolated from *D. andersoni* ticks provided by Dr. R. R. Parker and D. B. Lackman, of the Rocky Mountain spotted fever laboratory, Hamilton, Mont. was employed. Material was rewashed with ether and rickettsiae separated by differential centrifugalization in saline prior to use. Results showed that the average measurements of *R. rickettsi* were 1.2 μ by 0.6 μ.

In view of recent serological evidence that has indicated the antigenic relationship of *R. akari* to *R. rickettsi*, a comparison is of interest in that these two organisms also resemble one another closely (see Figs. 5 and 6).

Discussion

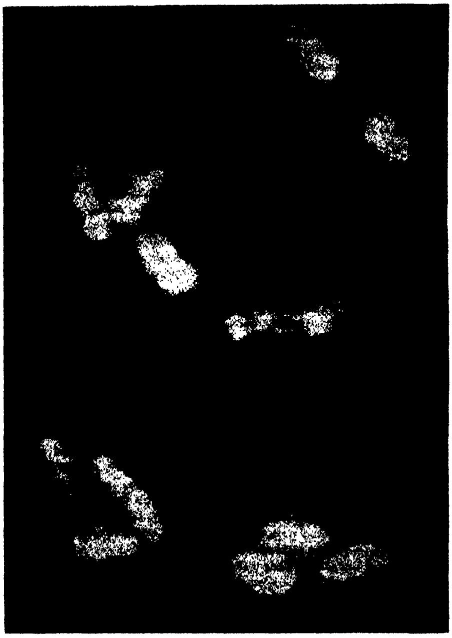
Three aspects of the biology of rickettsiae arise. Previously from unshadow-cast photographs of the rickettsiae of epidemic, murine, Rocky Mountain spotted fever, and Q. fever, Plotz *et al.* (3) concluded that it was not possible to distinguish one variety from the other. Our results reveal after shadow-casting that differences do exist in the average sizes and structure of five strains of rickettsiae studied. Secondly, it has been shown that rickettsiae, cultivated in the yolk sac of the embryonated egg and extracted by washing with ethyl ether followed by differential centrifugation (Craigie (1)), yield preparations that may be readily photographed with the electron microscope. There is no obvious evidence to suggest that ether (at low temperature) exerts a destructive effect on the outer covering or envelope of rickettsiae.

In their studies on the nature of the soluble antigen from typhus rickettsiae, Shepard and Wyckoff (5) concluded that so-called soluble antigen of typhus consisted of submicroscopic particles of capsular substance. We have noticed what probably are these same particles but our results show that they are derived from the central body of the rickettsia and not the capsule.

The third issue, arising out of this research, raises the important question of the relationship of the rickettsiae to the bacteria on the one hand and the viruses in general and psittacosis in particular.

During the course of studies on variola vaccinia virus, van Rooyen and Scott (4), despite careful search, failed to find clearcut or convincing signs that virus elementary bodies divided by binary fission. The present work on the rickettsiae suggests that it is occasionally possible to photograph rickettsiae showing central constriction analogous to bacterial binary fission. Much further research is necessary before these points can be clarified but we believe that the rickettsiae show morphological resemblances more closely related to the bacteria than the viruses. The morphological relationship of the rickettsiae to the psittacosis group of viruses is in course of investigation.

PLATE III



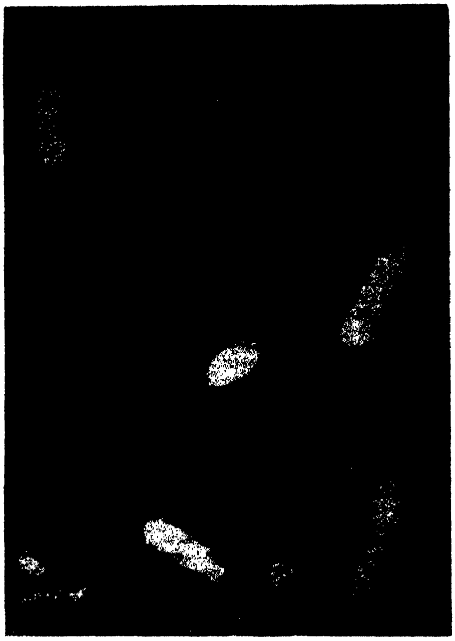


FIG. 5. *R. akari.* ×20,000

FIG. 6. *R. rickettsi.* ×20,000

All specimens shown have been shadowcast with chromium; the shadow to height ratio is approximately 4 : 1.

Conclusions

(1) Electron microscope photographs of *R. prowazeki*, *R. mooseri*, *R. rickettsi*, *R. akari*, and *R. burneti* have revealed certain differences in size and other morphological features.

(2) *R. burneti* has revealed internal structure of a pattern more striking than in other rickettsiae examined.

(3) *R. rickettsi* and *R. akari* appear to be morphologically similar.

(4) Structurally, the rickettsiae resemble the bacteria more closely than the viruses.

(5) Evidence of binary fission has been reported in rickettsiae.

Acknowledgments

Our thanks are due to Mr. D. R. Welch for the care and patience exercised in the preparation of illustrations, also to Miss Margaret Ranney for considerable technical assistance.

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THE DETECTION AND ASSAY OF HYALURONIDASE BY MEANS OF MUCOID STREPTOCOCCI[1]

By R. G. E. Murray and R. H. Pearce

Abstract

A method is presented by which hyaluronidase may be detected by observing its action on the colony characteristics of mucoid strains of *Streptococcus pyogenes* and certain strains of *Streptococcus* of Lancefield Group C growing on agar. This effect is due to destruction of the capsules of these organisms and is caused by hyaluronidase from all sources tried. Bacteria are tested for hyaluronidase production by streaking on seeded plates; fluids are tested by using assay cylinders or filter paper disks.

The method is convenient and has the advantage of detecting small concentrations of the enzyme, to a limit of between 0.001 and 0.0001 turbidity reduction units per ml. The test may be elaborated to give a means of biological assay that may be used where there are substances in the medium or diluting fluid that interfere with assays by other methods.

Provided the colonies do not autolyze during the 12 to 18 hr. required for the test, all mucoid *S. pyogenes* strains are suitable. However, among the Group C strains one was found with a capsule not destroyed by hyaluronidase, indicating a separate capsular type.

In testing certain genera for hyaluronidase production, notably *Streptococcus* and *Micrococcus*, antibiotic activity may interfere with the test. These inhibitory strains are relatively infrequent.

Introduction

The biological importance of the mucolytic enzyme, hyaluronidase, is well known because of its relationship to the "spreading factor", and has been reviewed thoroughly by Duran-Reynals (3) and Meyer (13).

The work of Seastone (19, 20), Kendall *et al.* (8), and Meyer *et al.* (14) has shown that the capsules of mucoid *Streptococcus pyogenes* and Group C streptococci consist of a complex polysaccharide chemically indistinguishable from hyaluronic acid. The nature of this capsular polysaccharide was further elucidated by McClean (10, 11) and Hirst (7) who demonstrated that hyaluronidase from testis and from the leech was capable of decapsulating completely these strains of mucoid streptococci.

One of us observed that the colony form of mucoid *S. pyogenes* is altered when growing in the neighborhood of colonies of *Micrococcus pyogenes* var. *aureus*, *Diplococcus pneumoniae*, and other hyaluronidase-producing organisms. This suggested a possible test and assay method for hyaluronidase using methods similar to those employed for the detection and assay of antibiotics, with mucoid streptococci as the indicator of enzyme activity.

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[1] *Manuscript received March 28, 1949.*

Contribution from the Department of Bacteriology and Immunology and the Department of Pathological Chemistry, University of Western Ontario, London, Ont. Presented in part at the meeting of the Laboratory Section, Canadian Public Health Association, Montreal, December, 1946 (15).

Materials

The Medium

The medium used for maintaining cultures and for the test is made as follows:

Proteose peptone No. 2 (Difco)	0.5 gm.
Proteose peptone No. 3 (Difco)	0.5 gm.
Sodium chloride	0.65 gm.
Agar (granulated, Agar Products Ltd.)	1.2 gm.
Papain broth (1) (pH 7.2)	add to 100 ml.

Four ml. of fresh, sterile, defibrinated sheep blood is added to each 100 ml. of the medium after autoclaving at 121° C. for 20 min. Thirty ml. of the medium in a 10 cm. plate provides a sufficient depth for the maximal growth of the streptococci.

The choice of a papain digest broth* may be fortunate since Rogers (16) showed that the hyaluronidase production of organisms was enhanced by a similar digest although different supplements were used.

The Substrate Organisms

A Lancefield Group A, type 9, *S. pyogenes* ("M. Str. 9"), was used for the majority of these experiments. Other strains, including members of the Lancefield Group C streptococci, were found to be satisfactory. The choice of suitable strains for this test is discussed in a later section of this paper. A number of the strains have been maintained for a period of over two years without change of character.

Methods

To detect hyaluronidase production by an organism, mucoid streptococci are spread on a plate to give confluent growth at the top and closely spaced but discrete colonies at the bottom. The organism under test is then streaked from top to bottom of the plate to give a confluent line of growth (see Fig. 1).

To detect hyaluronidase in a fluid, the fluid is placed in a penicillin assay cylinder or taken up by a filter paper disk, which is then placed on the surface of the seeded medium. A confluent growth of the mucoid streptococci over the entire surface of the plate is preferred. This may be achieved either by flooding the plate with a broth culture, aspirating the excess and drying, or more simply by spreading a streak of inoculum evenly over the surface of the plate (see Fig. 3).

The plates are incubated at 37° C. for 14 to 18 hr. Suitable strains of mucoid streptococci give good colony morphology under aerobic or anaerobic conditions, with lowered oxygen or increased carbon dioxide tension. Consequently, the conditions most suited to the growth and hyaluronidase production of organisms being tested may be chosen. However, for the testing of fluids, the most consistent results were obtained very simply in a "candle jar" (a desiccator kept in the incubator in which a candle is left burning when the jar is closed).

Standard Solutions of Hyaluronidase

Enzyme preparations were standardized by means of the turbidity reduction method of assay (9, 17), which is based on the turbidity that is produced by low concentrations of hyaluronic acid in the presence of serum at pH 4.2. One turbidity reduction unit (T.R.U.) is defined as that concentration of the enzyme which, under certain standardized conditions, will reduce the turbidity produced by 0.2 mgm. per ml. of sodium hyaluronate to that produced by 0.1 mgm. The hyaluronidase used as reference during this investigation was a partially purified extract of bovine testes containing approximately 10 T.R.U. per mgm. For use the enzyme was dissolved in normal saline.

Observations

The Effect of Hyaluronidase on Colonial Morphology

A streak of *M. pyogenes* is made with a small loop from top to bottom of a plate seeded with a mucoid *Streptococcus*. After 16 hr. incubation the streak is a confluent line about 3 to 4 mm. across, and the *Streptococcus* colonies are very mucoid. Around the streak there is a zone about 20 mm. across in which the colony morphology of the streptococcus is definitely altered (Fig. 1).

For 2 to 3 mm. on each side of the streak the colonies are small, high convex, and the surface has lost the glossy appearance of the mucoid colonies. At the outer margin of this zone the colonies flatten out and become larger flat disks on the surface of the medium, covering almost the same area as the normal mucoid colony. The boundary of this zone is very sharp, so much so that colonies are often seen to be cut in half: one half flattened and the other mucoid. In the region of confluent growth (not shown clearly in the figure) the boundary of the outer zone is sharp and resembles a step down from a plateau of mucoid growth to a lower level of less glossy growth. The inner zone previously described is seen as a narrow region of rougher, almost matt surface right up to the edge of the streak. The same picture was given when other strains of *M. pyogenes*, virulent strains of *Diplococcus pneumoniae*, certain strains of haemolytic *Streptococcus* of various groups, and other organisms were tested as set forth in a later section. Ultrafiltrates of broth cultures of *M. pyogenes* and of *D. pneumoniae*, readjusted to pH 7.0 or not, gave a similar effect when placed in assay cylinders. By the turbidity reduction method these preparations were shown to depolymerize hyaluronic acid.

A partially purified bovine testicular extract known to contain 10 T.R.U. of enzyme per mgm. was used to show that hyaluronidase produced this effect. Enzyme solutions made up in 1 to 4 mgm. per ml. concentrations in saline were put into penicillin assay cylinders on the surface of seeded plates. The testicular enzyme gave results exactly similar to those obtained with *M. pyogenes* (Fig. 2).

Colonies subcultured from either zone gave normal mucoid growth on fresh plates as did the mucoid colonies. Smears from areas of normal growth, and

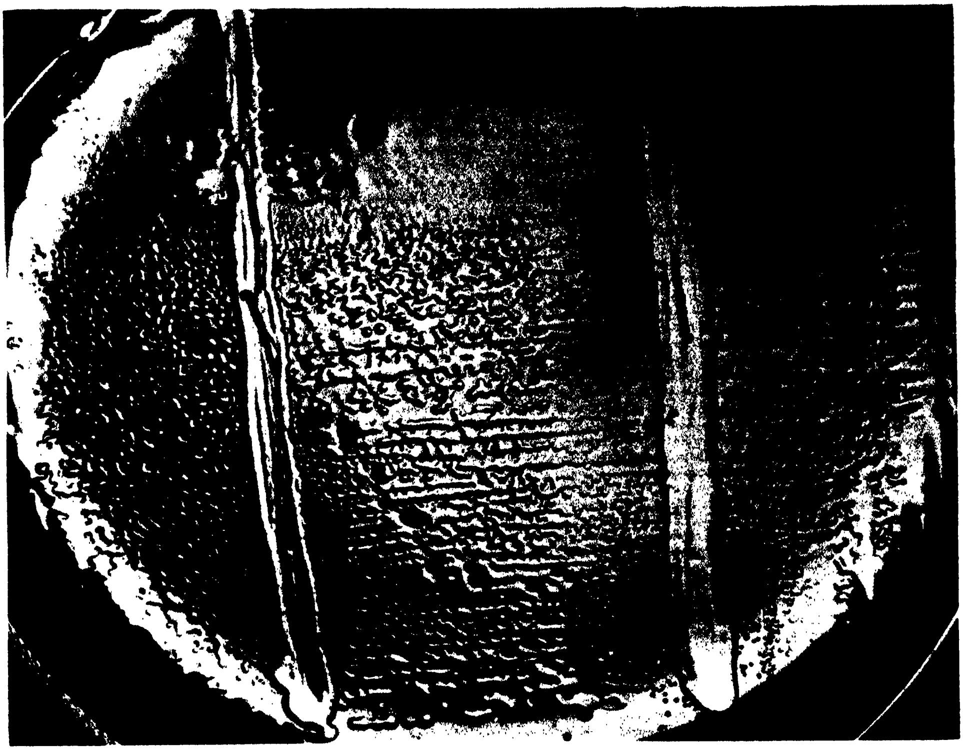
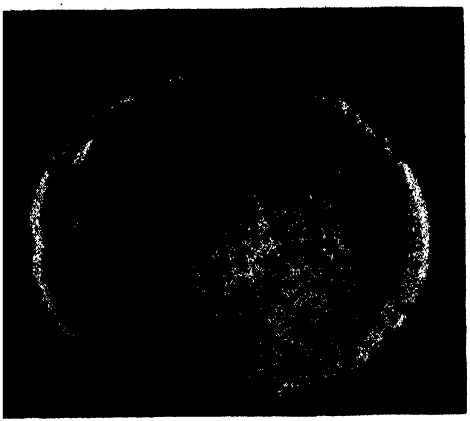


FIG. 1. *A photograph of a Petri dish spread with M. Str. 9 and streaked with two Micrococcus strains. The streak on the right is Micrococcus pyogenes var. aureus surrounded by the hyaluronidase effect. The streak on the left is a white Micrococcus sp. and does not show any effect.*

PLATE II



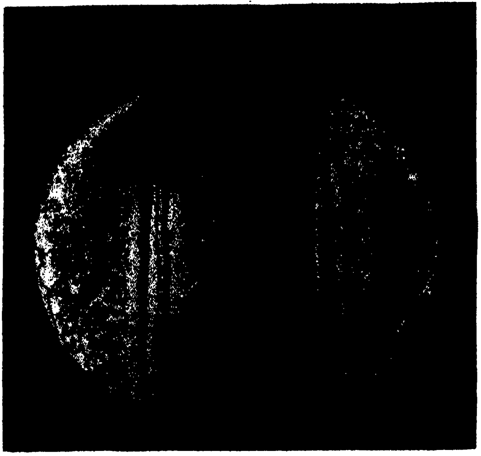


FIG. 2. *A photograph showing the effect of bovine testicular hyaluronidase on M. Str. 9. The cylinders contained fluid with approximately 1 T.R.U. per ml. of enzyme. The two zones may be seen, especially at the left.*

FIG. 3. *A photograph to show the effect of hyaluronidase on a confluent growth of M. Str. 9. This is evident around the upper and center cylinders.*

FIG. 4. *A photograph showing the inhibition of M. Str. 9 by two strains of Micrococcus pyogenes. The streak on the left shows a pure inhibition while that on the right shows inhibition with a superimposed hyaluronidase effect.*

from each of the two zones were examined for the size of capsules using the India ink method. Little difference was seen between organisms from the normal colony and the outer flattened zone, although the capsules seemed a little smaller in the latter. However, capsules of organisms from the inner (small colony) zone were absent or were definitely smaller when present. No differences were seen in the bacterial cells from the different zones. These observations were true for both the streak plates and the assay cylinder plates.

Neither saline nor phosphate buffers of pH values of 5, 6, 7, and 8 reproduced the effects seen with hyaluronidase preparations. Minor variations in the pH of fluids tested may have little effect because of the considerable intrinsic buffering power of the medium through which the tested fluid must diffuse. The ability to produce the effect is lost by heating the enzyme preparation to 100° C. Ascorbic acid, which shows definite spreading activity *in vivo*, does not show any flattening effect on colonies of mucoid streptococci in concentrations from 0.01 to 1.0% tested in the same manner as enzyme preparations.

That it is not an inhibition of the growth of streptococci can be shown by seeding a plate with substrate organisms and placing an absorbent disk (13 mm. diameter) saturated with an enzyme preparation of 1 T.R.U. per ml. After 12 hr. incubation, reading is made of the zone about the disk and an identical disk of the same enzyme concentration is placed on another portion of the plate. Readings are taken at intervals thereafter. A typical flattening effect is obtained and results are shown graphically in Fig. 5. The fact that, for the lower curve, the effect is not prevented because the mucoid colonies are already formed helps to substantiate the assumption that hyaluronidase acts on the capsules. The zone is smaller due presumably to the increased substrate concentration and the diffusion time. In addition, hyaluronidase preparations do not affect the growth of nonmucoid *S. pyogenes* of the same types as the mucoid strains employed.

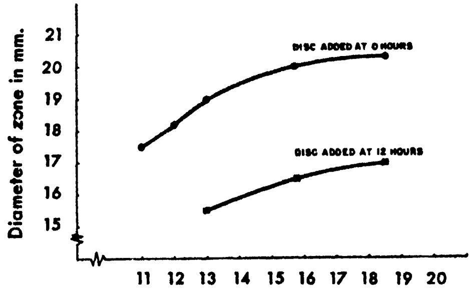


FIG. 5. *The effect of hyaluronidase when applied at different stages of growth.*

The Effect of Various Dilutions of an Enzyme Preparation

Serial dilutions of a standardized enzyme preparation were made in saline and pipetted into penicillin assay cylinders on plates seeded to give confluent growth of the mucoid streptococcus. Following incubation, the overall diameter of the effect was measured with vernier calipers as accurately as possible. Above a certain concentration of enzyme (ca. 1 T.R.U. per ml.) the size of the effect was constant or increased only very little whereas below that concentration the diameter of the effect decreased very rapidly. A representative experiment is shown graphically in Fig. 6. Since, in nearly all cases, there is a very sharp transition from the flattened zone to the normal mucoid growth this measurement may be made with reasonable accuracy. Plates were spread for confluent growth of the streptococci because of the ease of measurement of the sharply outlined zone; the appearance is shown in Fig. 3.

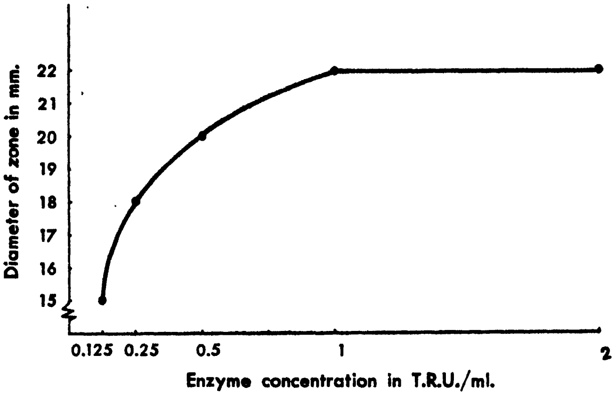


FIG. 6. *The relationship of the concentration of hyaluronidase to the diameter of the effect.*

The Detection of Low Concentrations of Enzyme

In seven replicates, using assay cylinders (on separate batches of medium and using different lots of enzyme), 0.001 T.R.U. per ml. produced an observable effect in each case; 0.0005 T.R.U. per ml. produced an observable effect in five cases and 0.0001 T.R.U. per ml. produced an observable effect in three cases. Using filter paper disks (½ in. in diameter) the lowest detectable concentrations of enzyme were 10 to 100 times greater than those detected using assay cylinders in parallel tests. For tests with low enzyme concentration the assay cylinders are the most satisfactory.

The rate of diffusion of hyaluronidase, and the growth rate and the capsular stability of the mucoid streptococci have great influence on the sensitivity of the test, especially when trying to detect low concentrations of hyaluronidase. Diffusion of the enzyme under the conditions of these experiments reaches a maximum after approximately 18 hr. (Fig. 5) in the incubator. On the other hand the colonies of mucoid streptococci tend to collapse after 12 to 24 hr. incubation, varying with the strain, and after this collapse reading of the test is impossible. The strain (M. Str. 9) that we have selected for these tests remains in good mucoid state for at least 18 hr. However, with enzyme concentrations less than 0.01 T.R.U. per ml., more reliable results may be obtained by placing the seeded plate, with the cylinders filled and in place, in the refrigerator for six hours before incubating so that diffusion may take place.

Use as a Biological Assay

From examination of the curves obtained by plotting the diameter of the effect against the concentration of enzyme as in Fig. 6, it seemed likely that a biological assay might be developed on the same basis as plate assays for penicillin and other antibiotics. It was found that the curves could be made linear if plotted on semilog paper, as shown in Fig. 7. This linear relationship applies only to concentrations of hyaluronidase of 1 T.R.U. per ml. or less.

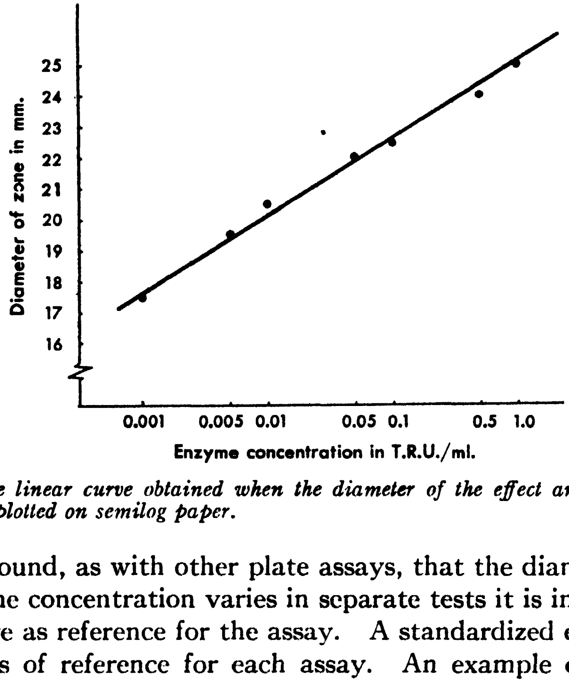


FIG. 7. *The linear curve obtained when the diameter of the effect and the enzyme concentration are plotted on semilog paper.*

Since it is found, as with other plate assays, that the diameter of effect for a given enzyme concentration varies in separate tests it is impossible to use a standard curve as reference for the assay. A standardized enzyme is used to provide points of reference for each assay. An example of an attempt at assay is given graphically in Fig. 8. In this graph the "unknown" has been assigned an arbitrary position. It is essential to such an assay that the slopes of the curves for "unknown" and "standard" enzyme preparations be as nearly identical as possible. As can be seen in Fig. 8 the parallelism is reasonably good. Knowing the dilution factors for the "unknown" the assay can be made by direct projection on the graph.

The work done on this assay is only adequate to show that the method is feasible. It would probably be worthwhile only for enzyme concentrations in the range of 1.0 to 0.005 T.R.U. per ml. and, for improvement of the accuracy, would require an even layer of medium on a flat surface large enough to take all the cylinders or disks required for both "unknown" and "standard".

Application to Tissue Extracts

The qualitative test is useful for detecting hyaluronidase activity in tissues and fractions of tissue extracts. A block of tissue placed on the seeded medium shows little or no hyaluronidase activity. But extracts of tissue, such as testis, ground in a Latapie Mill or in a mortar with sand using a small volume of fluid, can be tested by the described techniques. The method was used successfully to follow activity in preparations from human, bovine, and rat testes. Whether enzyme inhibitors, such as those described by Haas (5) and others, would interfere with the testing of tissues and tissue fluids has not been investigated. However, trials were made with four hyaluronidase inhibitors (hyaluronic acid derivatives) described by Hadidian and Pirie (6) and also with heparin in the diluent fluid. These slowed the viscosity reduction of hyaluronate in the viscosimeter from two to five times. Using the plate test, no significant differences were observed between the controls and solutions containing the inhibitor, possibly because of the time relationship involved in incubation of plates for 12 to 18 hr. and of reversal due to the elevated substrate concentration.

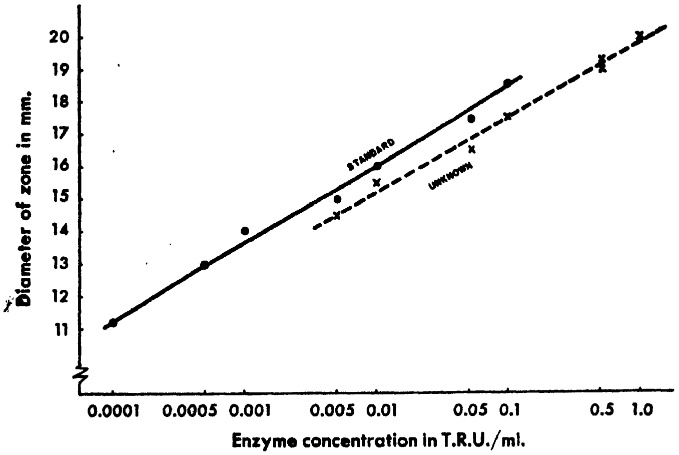


FIG. 8. *An example of the biological assay of hyaluronidase. The unknown is given an arbitrary position in the graph and is referred to the curve of a standard enzyme preparation.*

Application to Bacteria

Using the streak plate test an extensive survey of organisms could be carried out in short time, though no such attempt was made here. The plates may be read after 12 to 14 hr. incubation and are good after an average "overnight" incubation if a suitable mucoid *Streptococcus* strain is used. The zone of flattening of colonies is always well defined and rarely comes so close to the streak that the result must be recorded as doubtful. This makes the test simple and certainly not laborious. In view of the sensitivity of the test and the tolerance of the mucoid streptococci for varied gaseous conditions, it would seem to be a suitable method for determining whether an organism does or does not produce hyaluronidase.

A survey of available strains, most of them freshly isolated, is shown in Table I. It can be seen that, in general, the organisms known to produce

TABLE I

A SURVEY OF ORGANISMS FOR HYALURONIDASE PRODUCTION USING THE PLATE TEST

Organisms tested	No. of strains tested	Not producing hyaluronidase	Producing hyaluronidase	Doubtful
Streptococcus pyogenes (Group A)	17	5	12	0
Streptococcus (haemolytic ? Group A)	6	2	4	0
Streptococcus pyogenes (mucoid)	12	12	0	0
Streptococcus (Group B)	2	1	1	0
Streptococcus (Group C, human)	1	0	1	0
Streptococcus (Group C, "animal pyogenes")	7	7	0	0
Streptococcus (minute, haemolytic, Group G)	1	0	1	0
Streptococcus (Group H)	1	0	1	0
Streptococcus faecalis	2	1	0	1
Streptococcus (*viridans* group)	7	6	1	0
Diplococcus pneumoniae	12	0	12	0
Micrococcus pyogenes	113	0	112	1
Micrococcus sp. (coagulase negative)	6	6	0	0
Klebsiella pneumoniae Type A	1	0	0	1
Type B	1	1	0	0
Corynebacterium diphtheriae	6	6	0	0
Clostridium welchii	1	0	1	0
Clostridium histolyticum	1	1	0	0
Clostridium septique	1	0	1	0

hyaluronidase give the characteristic changes on mucoid streptococci. The streptococci (45 strains from Groups A, B, and C) show the known variability of hyaluronidase production as exemplified in the work of Crowley (2). In Crowley's series, hyaluronidase production in Group A was restricted to types 4 and 22 to which we may add types 9 and 19, although not all our strains were typed. Sherwood (22) has used this plate test on a large number of strains of streptococci of various groups and found "complete correlation" with viscosimetric and mucin clot prevention methods of detecting hyaluronidase. The capsulated virulent pneumococci were all active, but one type II strain dissociated to rough and lost the ability to produce the enzyme. Among the micrococci the positive correlation of hyaluronidase production and the production of plasma coagulase shown by Schwerbacher *et al.* (18) is supported, with an indication that the correlation may be better than they reported.

An antibiotic effect produced by certain organisms may interfere, but in our experience the frequency of these strains is low. Sherwood (21, 22) has described representatives from nine Lancefield Groups that produce an antibiotic inhibition of one or more other strains of β-haemolytic streptococci including the M. Str. 9 used in our tests. Three of Sherwood's strains were found to inhibit all of our strains of mucoid Group A and Group C streptococci to varying degrees. However, on those where inhibition was very slight, there was no evidence of a superimposed hyaluronidase effect. Out of the 113 strains of *M. pyogenes* examined, three were found to have an inhibitory effect. Of these three, two produced a hyaluronidase effect much wider than the inhibition (see Fig. 4), which was easily recognized. The remaining strain did not inhibit two other strains of mucoid *S. pyogenes* and on these it gave a very narrow and doubtful zone and is recorded as such in Table I. These inhibitors are being investigated.

Qualitative tests may be carried out with broth cultures, although with less convenience, and using the projected method of biological assay it is possible to get a figure equivalent to T.R.U. per ml. for the medium in which the organism is grown. All cells must be removed from the medium or their growth will give altered results. Using Corning fritted glass U.F. filters we have obtained good results providing at least 10 ml. is filtered, after preliminary clarification by centrifugation. Lower activity is obtained with smaller amounts, presumably because of some adsorption to the filter. These tests have been hard to control because of the effect on the turbidity reduction assay of various constituents (? peptones) of the complex media required for adequate growth and enzyme production by the pneumococci that were used in these experiments.

To find out whether some media in common use would effect the test when used as diluents, an enzyme preparation of 5.0 T.R.U. per ml. was diluted 10 times with each medium under test and placed in three assay cylinders (Table II).

TABLE II

Diluent	Mean zone diameter of 3 cylinders, mm.
1. Normal saline	18.3
2. Peptone water	18.7
3. Papain broth	18.5
4. Dextrose broth	16.7
5. Brewers medium	15.5
6. Litmus milk	15.0

There is a significant difference between the two main groups, 1, 2, and 3 and 5 and 6, with 4 in an intermediate position. These differences are probably not due to inhibition of the enzyme but, much more likely, to the presence of fermentable carbohydrate stimulating the growth of the mucoid *Streptococcus*. This stimulation was evident in the controls and, causing an increase in the amount of substrate to be hydrolyzed, may be expected to give the deviation of readings observed. The medium in use must, therefore, be considered as possibly deflecting the sensitivity of the test especially if it contains fermentable carbohydrate.

The Choice of Mucoid Strains as Substrate Organisms

Strains of *S. pyogenes* with an obviously mucoid appearance are of comparative rarity in clinical bacteriology. They have a large colony form that is rather watery in appearance, and, when fished, have a very sticky mucinous character. On examining a number of strains thought to be mucoid on primary isolation the sticky colony grades almost insensibly to colonies of the same order of appearance but of a pasty character when fished, although they all have capsules of varying size at some time during growth. Many of the latter strains were found to produce hyaluronidase whereas none of the 12 truly mucoid strains produced the enzyme. Among the 12 strains, four were unsuitable as substrate organisms because the colonies flatten too quickly or because of a tendency to dissociate. Our original strain, M. Str. 9, proved the most satisfactory together with a probably identical strain, 1175 A9. Stability of the mucoid colony and exuberant growth are the most necessary characteristics of suitable strains for this test.

Strains of *Streptococcus* ("animal pyogenes") Lancefield Group C have a very persistent mucoid state on incubation and should be, theoretically, suitable for this test. Seven strains were tried. None of them produce the hyaluronidase effect on the suitable *S. pyogenes* strains. Six strains were affected by both bacterial and testicular hyaluronidases, though some were more suitable than others because of more exuberant growth. Strain 34406 C, however, despite its grouping and excellent capsulation is not affected by bovine or human testicular extract nor by different strains of known hyaluronidase producing bacteria (Group A streptococci, pneumococci, and micrococci). This is evidence that there may be two types of mucoid strains—with capsular polysaccharides sensitive and not sensitive to hyaluronidase.

Discussion

The methods used for the detection and assay of hyaluronidase (13) are beset with many difficulties and disadvantages. Chemical methods are not yet sufficiently specific, simple, and sensitive enough to be of value for routine use. Physical methods involving viscosity and turbidity require rigidly controlled conditions, special apparatus, and the isolation of hyaluronate. The mucin clot prevention test is sensitive but often at variance, quantitatively, when referred to other means of assay (12). Biological methods using skin diffusion are expensive, subject to variability, and other "spreading" substances may be present in preparations. The physical methods are the most used and the most reliable. However, for some work they are unsatisfactory because they are not particularly sensitive to low concentrations of enzyme.

A test, based on the decapsulation of Group C streptococci by hyaluronidase, was reported recently by Fulton, Marcus, and Robinson (4). The degree of decapsulation is determined by observing changes in the packed cell volume of a suspension treated with various concentrations of enzyme. The method has the advantage of speed and is sensitive down to 0.01 viscosity reducing units* per ml., but has the disadvantage of requiring considerable manipulation. The biological method described in this paper provides a qualitative test that requires for its success only the provision of adequate growth conditions for a mucoid *Streptococcus*. The fact that it is very sensitive and will detect a minimum concentration of between 0.001 and 0.0001 T.R.U. per ml. is a point in its favor. A minimum of manipulation is required. It is also relatively unaffected by the presence of extraneous substances such as those found in media. The method can be applied as a simple test for the production of hyaluronidase by growing bacteria and to the detection of the enzyme in both crude and purified tissue extracts. Although it is feasible it does not seem necessary to elaborate the test as a means of biological assay until a need arises.

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* *Viscosity reducing unit equivalent to two turbidity reducing units.*

Acknowledgments

The authors are indebted to Dr. G. G. Kalz (McGill University, Montreal), Dr. E. T. Bynoe (Laboratory of Hygiene, Ottawa), and Dr. R. M. Pike (Southwestern Medical College, Dallas, Texas) for the cultures they supplied. Dr. Noble P. Sherwood (University of Kansas, Lawrence, Kans.) was most generous in supplying inhibitory strains of streptococci and kindly undertook a trial of the method. Dr. N. W. Pirie (Rothamsted Experimental Station, England) was kind enough to provide samples of hyaluronidase inhibitors. The Schering Corporation, Plainfield, N.J., supplied generous amounts of purified hyaluronidase, through the courtesy of Dr. Erwin Schwenk. We would like also to thank many colleagues, especially Dr. I. N. Asheshov, for aid and counsel. The photographs used in the paper were taken by Mr. W. Austin of the Art Service, University of Western Ontario.

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THE MICROCOLORIMETRIC DETERMINATION OF INORGANIC PHOSPHATE IN PLASMA AND URINE[1]

I. QUANTITATIVE INVESTIGATION OF PRESENT COMMON PROCEDURES

II. A SENSITIVE AND STABLE METHOD FOR PLASMA, SERUM, AND URINE

BY JOHN R. POLLEY[2]

Abstract

A quantitative investigation of the common colorimetric procedures used for determining inorganic phosphate was made to determine the factors affecting the sensitivity and color stability. A method is presented that is accurate to within ±2% and is sufficiently sensitive to give quantitative color development with less than 1γ of phosphorus. The color produced is quite stable for colorimetry. The method has been adapted to the determination of phosphorus in 0.1 or 0.02 ml. of plasma, serum, or urine or in the protein-free filtrate residue from the determination of sodium, potassium, calcium, and chloride in 0.4 ml. of plasma.

Phosphorus is present in the blood to the extent of about 40 mgm. %, mainly in the forms of lipid phosphorus (12 mgm. %), inorganic phosphate (5 mgm. %), and organic acid-soluble phosphate esters (25 mgm. %). For the quantitative determination of these different forms, they are first separated on the basis of their physical chemical properties. The phosphorus content in each case is then determined by first converting the phosphorus to inorganic phosphate by ashing, where necessary.

A method for the microdetermination of sodium, potassium, calcium, and chloride in a single plasma sample was published recently (8). In this procedure, 0.4 ml. of plasma is added to 3.5 ml. of distilled water, followed by the addition of 1.0 ml. of 15% trichloroacetic acid. After centrifuging, about 4.7 ml. of protein-free filtrate is obtained by decanting the supernatant liquid, 1.0 ml. aliquots being used for each of the above analyses. It was proposed to devise a method for the determination of inorganic phosphate on 0.5 ml. of the remaining filtrate.

The differences between the various methods for phosphorus determination lie in their treatment of the inorganic phosphate solutions. Most procedures in present use are variations of two older methods that differed in the reducing agent used: 1-amino-2-naphthol-4-sulphonic acid in the method of Fiske and Subbarow (4), stannous chloride in the method of Kuttner and Cohen (6).

The use of 1-amino-2-naphthol-4-sulphonic acid was proposed by Fiske and Subbarow as a reducing agent instead of the hydroquinone previously used by Bell and Doisy (1). They found that this reagent rapidly reduced the phosphomolybdic acid at room temperature and imparted the advantages that the intensity of the blue color produced was less affected by pH differences in the

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[1] *Manuscript received May 21, 1949.*

Contribution from the Department of Medical Research, University of Western Ontario, London, Canada.

[2] *This work was done during the tenure of a Life Insurance Medical Research Fellowship.*

solutions and was more stable for colorimetry than that given by hydroquinone. Roe and Whitmore (10) simplified the method and the preparation of reagents.

Stannous chloride as reducing agent was introduced by Kuttner and Cohen (6), the method being modified later by Kuttner and Lichtenstein (7), for the specific reduction of the phosphomolybdic acid. They found that the intensity of the blue colored solution was about 10 times that of the Fiske and Subbarow method and was proportional to the phosphorus content. However, it was found that with the molybdic acid – stannous chloride reagent, an excess of acid decreased color development, whereas an excess of sodium molybdate increased the color intensity but also increased the color in the reagent blank. Using a modified preparation of the reagents, Shinowara, Jones, and Reinhart (11) found that by reducing the acidity of the protein-free filtrate, the effect of trichloroacetic acid on the diminution of color development described by Bodansky (3) was completely negated. The method has the disadvantage that the color intensity changes with time.

In 1938, Berenblum and Chain (2) devised a method in which the reducible phosphomolybdic acid is extracted from the aqueous solution with isobutyl alcohol and then reduced with stannous chloride. This method is less sensitive to changes in acidity, concentration of reagents, and interfering substances than those previously described.

Kitson and Mellon (5) described a method for the determination of phosphorus in steels using ammonium vanadate rather than the previous reducing agents. A yellow colored solution of molybdivanadophosphoric acid is formed, the color intensity of which is determined colorimetrically as a measure of the phosphorus content. This method has been adapted to the determination of serum phosphate (12).

From these introductory remarks it can be seen that the stannous chloride method is considered to have the advantage of increased sensitivity but the color intensity changes with time and is somewhat pH dependent, whereas the use of 1-amino-2-naphthol-4-sulphonic acid or ammonium vanadate gives increased color stability but the color is less intense and so less sensitive.

A quantitative comparison of these different methods was made using the following experimental procedure and phosphate standards. The various experimental conditions such as pH and relative reagent concentrations were investigated in an attempt to develop a method possessing the optimum of color intensity and stability. In each case, the reagents were freshly prepared according to the authors' directions. The instrument used was an Evelyn photoelectric colorimeter with a 660 filter for the blue solutions.

Experimental

A tentative model of an experimental procedure was set up as follows:

(1) To 5 ml. of 5% trichloroacetic acid add 0.5 ml. of protein-free filtrate.

(2) Add 0.5 ml. of molybdate reagent.

(3) Add 0.5 ml. of reducing agent in five minutes.

1-Amino-2-naphthol-4-sulphonic acid as Reducing Agent

The reagents were prepared as outlined by Roe and Whitmore (10); 2.5% ammonium molybdate (Step 2 above), 250 mgm. % 1-amino-2-naphthol-4-sulphonic acid in sulphite–bisulphite solution as reducing agent (Step 3 above). The variables investigated were (1) ammonium molybdate concentration, (2) reducing agent concentration, (3) effect of trichloroacetic acid.

The effect of the concentration of the ammonium molybdate solution was investigated by preparing calibration curves between galvanometer readings and solutions of known phosphorus content over a range of concentrations of ammonium molybdate. In each case, a reagent blank was prepared and the instrument set to 100 with the blank in place (set to 0 with instruments having a semilogarithmic scale). The colored solutions were read from time to time to observe the stability. In Fig. 1 is shown the calibration curve obtained over a range of ammonium molybdate concentrations from 0.5 to 2.5%, the readings being made after five minutes. It can be seen that, over this range, the calibration curve is independent of the concentration of the ammonium molybdate solution. Below 0.5%, color fails to develop quantitatively. A disadvantage not apparent in Fig. 1 is shown in Fig. 2, which shows the effect of increasing ammonium molybdate concentration on the stability of the color produced with a given phosphate standard (2.5 mgm. % P) over a period of two hours. As the concentration of the ammonium molybdate solution is increased, the intensity of the color produced is increased but the color stability is decreased; the color intensity in all tubes, including the reagent blanks, increases and continues to increase with time. Despite this, up to a concentration of 2.5%, the calibration curve remains almost constant over a period of two hours when the reagent blank is set to 100 for each reading.

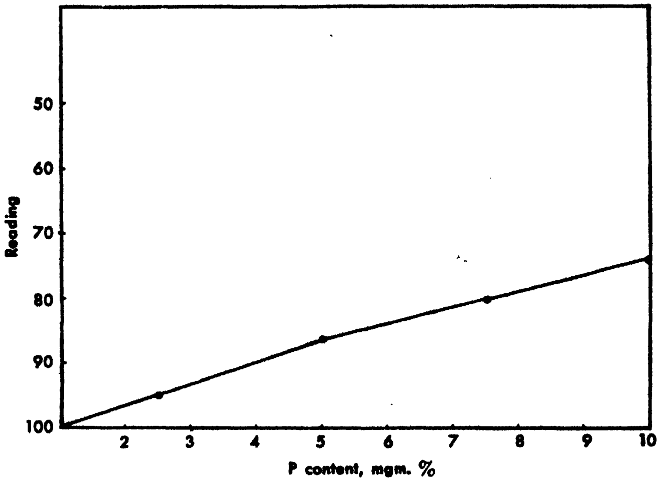

FIG. 1. *Calibration curve using Fiske and Subbarow method over a range of ammonium molybdate concentrations from 0.5 to 2.5%.*

Above 2.5% the color is so intense and unstable that it is impossible to construct an accurate calibration curve. At 0.5%, the reagent blank is colorless and the calibration curve is stable. Increasing the concentration of the ammonium molybdate solution above 0.5% does not increase the sensitivity of the method, since the increased color produced in the phosphate standards is negated by the increased color in the reagent blank and the color becomes more unstable.

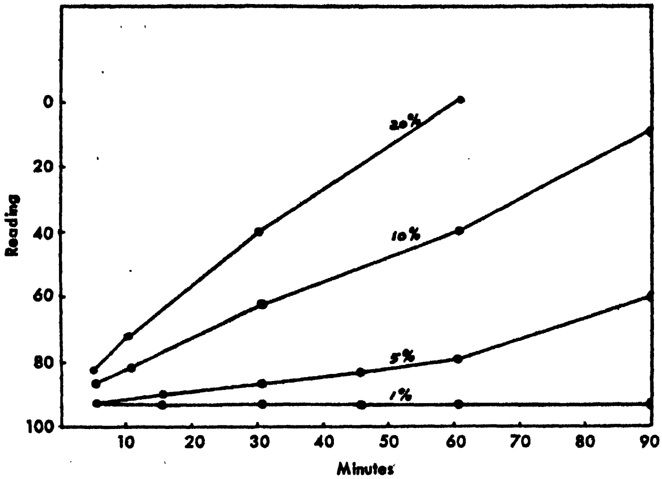

FIG. 2. *Effect of increasing ammonium molybdate concentration on color stability.*

In a similar manner, the effect of varying the concentration of the reducing agent was studied. It was found that as the concentration was increased from 50 to 550 mgm. %, the intensity of the color produced with a given phosphate standard increased, but so did the color in the reagent blank. When the correction for the reagent blank was made in each case, all tubes gave almost identical readings, showing that the net color intensity is not sensitive to changes in the reducing agent concentration over the tenfold range tested. When 10% ammonium molybdate was used instead of the 2.5%, the color intensity increased as the concentration of the reducing agent was decreased but in all cases the color was very unstable.

The effect of trichloroacetic acid was studied in a similar manner by varying the concentration of it used in Step 1. It was found that, over a range of concentration from 0.5 to 15%, the calibration curves obtained were almost identical. Likewise, the color stability was not affected by the amount of trichloroacetic acid present.

Briefly, this method is quite insensitive to trichloroacetic acid, relatively independent of the reducing reagent concentration over the range of 50 to 500 mgm. % tested, and independent of the ammonium molybdate concentration between 0.5 and 2.5% but seriously affected above this.

Stannous Chloride Method

The reagents were freshly prepared as outlined by Shinowara, Jones, and Reinhart (11); 3% molybdic acid—1 part of 7.5% sodium molybdate + 1 part of 10 *N* sulphuric acid (Step 2), 0.07% stannous chloride (Step 3).

The effect of varying the concentration of the molybdic acid was studied as before by preparing calibration curves over a range of molybdic acid concentrations. The tubes were read from time to time as a check on the color stability. It was found that over a range of concentrations from 1 to 10%, the calibration curves obtained at a given time interval after the addition of the last reagent did not differ by more than three galvanometer divisions (0.3 mgm. % P) at any point. Over a period of two hours, the color intensity faded slightly so that all the calibration curves showed a small drift. This drift, however, was not greater than five scale divisions (0.5 mgm. % P). Between 30 and 60 min. the color was almost completely stable. The intensity of the color produced with a given phosphate standard rose to a maximum with 1% molybdic acid; above this, there is no increase in the net color intensity. The color stability is unaffected by changing molybdic acid concentration. When the ratio of the sodium molybdate to sulphuric acid in the molybdic acid solution was increased threefold, color production rose to a maximum between 0.5 and 1% molybdic acid. Above this, the color produced in all tubes, including the reagent blanks, rapidly increased but the net color intensity with a given phosphate standard decreased.

The sensitivity of the method to the stannous chloride reagent was studied by analyzing a given phosphate standard over a range of stannous chloride concentrations. The color intensity in the standards and corresponding reagent blanks increased slowly as the concentration was increased from 0.005 to 0.1% and then increased more rapidly. There was no increase in the net color intensity over this large range, however. These results may possibly explain the criticism of this method expressed by Roe and Kahn (9) who claimed that the values obtained varied with the amount of stannous chloride reagent added. Thus, it is true that the intensity of color produced with a given phosphate standard increases slightly as the concentration of the stannous chloride is increased, but when the corresponding reagent blank is set to 100, there is no change in the net color intensity. The color stability was not affected by changing stannous chloride concentration.

The effect of trichloroacetic acid on the intensity and stability of the color produced with a given phosphate standard (2.5 mgm. % P) was studied by varying the concentration of it used in Step 1. The results are shown in Fig. 3, the colorimeter having been set to 100 with distilled water in place. It can be seen that the stability of the color after the first 10 min. is unaffected by the amount of trichloroacetic acid present. The intensity of the color produced increases as the concentration of trichloroacetic acid is decreased. This suggests that the sensitivity of the method is also increased by decreasing the amount of trichloroacetic acid present. This was the conclusion reached by Shinowara, Jones, and Reinhart (11) who recommended developing the color in a nearly neutral solution and reading in exactly six minutes since the color was unstable with time. A different conclusion is reached, however, when the complete results in Fig. 4 are considered. If the curves shown in Fig. 4 are made only five minutes after the addition of the stannous chloride reagent, there is a small increase in the net color intensity as the trichloroacetic acid concentration is decreased but this is before the color has reached its level of maximum stability. The curves shown in Fig. 4 and described below were obtained after 15 min. and showed a drift of only two to three scale divisions up to two hours. Three curves are shown; *A* represents the values obtained

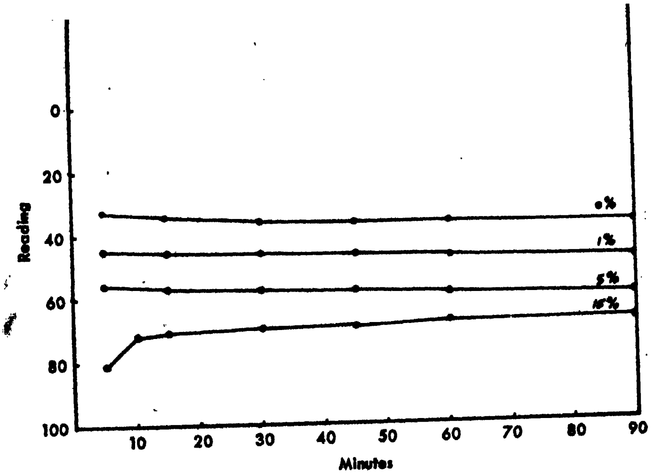

FIG. 3. *Effect of trichloroacetic acid concentration on color intensity and stability.*

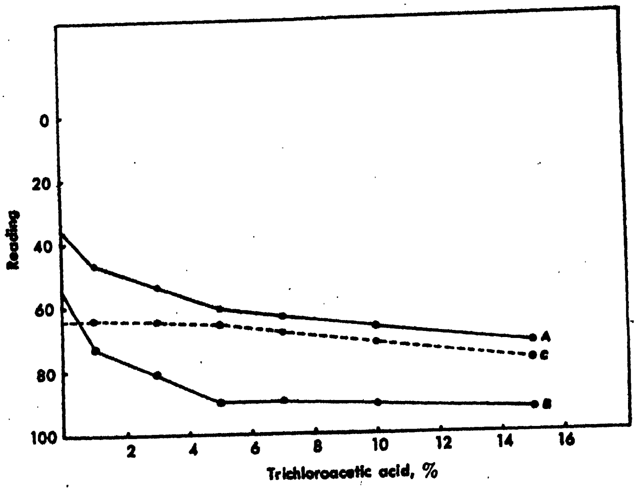

FIG. 4. *Effect of trichloroacetic acid concentration on absolute color* (*A*), *reagent blank* (*B*), *and net color* (*C*).

when the colorimeter is first set to 100 with distilled water in place, *B* is the color in the corresponding reagent blanks, and *C* is the curve obtained when the standards are read after the colorimeter has been set to 100 with the corresponding reagent blank in position. From Curve *A* it can be seen that the color intensity increases rapidly as the amount of trichloroacetic acid is reduced. This is the same effect shown in Fig. 3, apparently suggesting that the sensitivity of the method is also increased by decreasing the acidity. From Curve *B*, however, it is seen that the color in the reagent blanks is also greatly increased. Curve *C* shows that from 0 to 5% trichloroacetic acid, there is no increase in the net color intensity.

Thus, in the stannous chloride method, the net color intensity and stability are relatively independent of the molybdic acid concentration over the range 1 to 10% tested, independent of the stannous chloride concentration over the range 0.005 to 0.1% and independent of the trichloroacetic acid concentration over the range 0 to 5%. Under these conditions, this method is considerably more sensitive and no less stable than the method of Fiske and Subbarow.

Ammonium Vanadate Method

The reagents were freshly prepared as outlined by Simonsen *et al.* (12); 0.25% ammonium vanadate (Step 2), 5% ammonium molybdate (Step 3).

The effect of varying the concentration of the ammonium vanadate was studied in the usual manner. It was found that the intensity was unchanged over a concentration range of 0.1 to 1% and was completely stable over the two hour period tested. The color intensity, however, is inadequate for a micromethod under the present conditions.

The color intensity and stability were found to be independent of the amount of trichloroacetic acid over the range of 0 to 15% tested.

Increasing the concentration of ammonium molybdate over the range 1 to 20% caused an increase in the color of the phosphate standards and reagent blanks but did not increase the net color intensity.

The Fiske and Subbarow method using aminonaphtholsulphonic acid is widely used and is considered to have the advantage over the stannous chloride method of producing a color that is more stable although less intense. The use of stannous chloride, on the other hand, has the advantage of producing a greater color intensity and so an increased sensitivity; a disadvantage often cited is that the color is less stable and so the time factor must be carefully controlled.

This present investigation has shown that the ammonium vanadate method gives the greatest color stability but is not sufficiently sensitive for microbiological determinations. The Fiske and Subbarow method was found to be quite insensitive to the amounts of the various reagents present over a considerable range whose limits have been described quantitatively. The method can be considered to be stable, if by stable it is understood that the phosphate standards or unknowns give a fairly constant reading over a two hour period of time when the reagent blank is taken into account before each reading. The method is less stable, however, than is generally considered when a calibration curve prepared, for example, after five minutes, as is usually the case, is used to read the standards or unknowns at a later time. That is, the color in both standards and reagent blanks does undergo some change with time, a value constant to within 0.3 mgm. % P only being obtained when the reagent blank is taken into account at each time.

The stannous chloride method is equally insensitive to the reagents present under the conditions defined when the reagent blank is taken into account before each reading. Under the conditions defined, it is more sensitive than the Fiske and Subbarow method and no less stable. The use of sodium molybdate in the stannous chloride method has the disadvantage that it is alkaline and on standing in a bottle, forms silicates from the glass. Silicate reacts similarly to phosphate in this reaction and, as the sodium molybdate ages, the color in the reagent blank increases. Ammonium molybdate does not have this disadvantage.

It was therefore proposed to devise a method replacing sodium molybdate with ammonium molybdate for greater reagent stability and to use stannous chloride for the greatest sensitivity. After a series of investigations on the different reagents such as were conducted above, the following method was developed.

Reagents

(1) Molybdic acid reagent. (*A*) Dissolve 2.5 gm. of reagent-grade ammonium molybdate in 97.5 ml. of distilled water. If there is any turbidity, the solution should be filtered. (*B*) Dissolve 150 gm. of reagent-grade trichloroacetic acid in 850 ml. of distilled water. Then add (*A*) to (*B*), mix well, and store in a dark bottle in the cold. This reagent is stable.

(2) Stannous chloride, stock solution. Dissolve 6 gm. of reagent-grade stannous chloride in 10 ml. of concentrated hydrochloric acid. Store in a dark bottle in the cold. This reagent is quite stable.

(3) Stannous chloride, dilute analytical reagent. Add 0.2 ml. of the stock stannous chloride to a 100 ml. volumetric flask and make up to the mark with distilled water. This solution is not stable for more than about six hours and so should be made up only for immediate use.

Procedure

(1) To a colorimeter tube add 10.0 ml. of the molybdic acid reagent.

(2) Add 0.5 ml. of the protein-free filtrate prepared as described on Page 1 (1).

(3) In about five minutes, add 0.5 ml. of the freshly prepared dilute stannous chloride reagent.

(4) Read in 15 min. or later in a photoelectric colorimeter using a 660 mμ filter. The instrument is set to 0 (to 100 with an Evelyn colorimeter) with a reagent blank in place. The reagent blank is prepared as above except that 0.5 ml. of distilled water is used in Step 2 instead of protein-free

filtrate. The phosphorus content of the samples is read from a calibration curve between colorimeter readings and phosphorus content established by treating known solutions of monopotassium phosphate over the range 2.5 to 10 mgm. % of phosphorus by this procedure. The phosphorus standards are run at the same time as the unknowns.

TABLE I

ANALYSIS AND RECOVERY OF PHOSPHATE IN PLASMA

Sample	Phosphorus found, mgm. %	Phosphorus added, mgm. %	Phosphorus recovered, mgm. %
1	4.4	2.50	2.60
	4.5	2.50	2.55
	4.5	2.50	2.50
	4.5	2.50	2.55
2	5.6	5.00	5.10
	5.5	5.00	5.00
	5.6	5.00	5.10
	5.6	5.00	5.00

In Table I are shown the results of a typical analysis on samples of plasma, each sample being analyzed in quadruplicate to show the degree of precision of the method. Also shown are the results of recovery experiments, obtained by adding known amounts of inorganic phosphate to the previously analyzed samples.

From the above analyses it can be seen that the method is accurate within ±2%.

If only the inorganic phosphate level of plasma or serum is to be determined, a 0.1 ml. sample is used.

(1) Add 0.1 ml. of plasma or serum to 1.0 ml. of distilled water, rinsing the pipette by drawing the water up and down a few times.

(2) Add 1.0 ml. of 15% trichloroacetic acid, allow the mixture to stand for about 15 min. and then centrifuge.

(3) The analysis is performed as outlined in the procedure above except that 1.0 ml. of this protein-free supernatant is used in Step 2. The calibration curve is prepared by treating phosphorus standards in exactly the same manner as the plasma or serum.

If necessary, the analysis can be performed on a 0.02 ml. sample of plasma or serum as follows:

(1) Add 0.02 ml. of plasma or serum to 1.5 ml. of distilled water.

(2) Add 1.0 ml. of 15% trichloroacetic acid, allow the mixture to stand for about 15 min., and then centrifuge.

(3) Add 2 ml. of this protein-free supernatant to 5.0 ml. of the molybdic acid reagent.

(4) In about five minutes, add 0.5 ml. of the freshly prepared dilute stannous chloride reagent. The calibration curve is prepared by treating phosphorus standards in exactly the same manner as the plasma or serum.

The inorganic phosphate in urine can be determined by first diluting it 1 : 50 in 0.1% trichloroacetic acid. A 0.1 ml. sample is then treated in the same manner as 0.1 ml. of plasma or serum.

This method is simple and rapid and is accurate to within ±2%. It is sufficiently sensitive to give quantitative color development with less than 1γ of phosphorus. The color produced is quite stable for colorimetry; a calibration curve between colorimeter readings and standards of known phosphorus content prepared 15 min. after the addition of the stannous chloride reagent can be used to measure the phosphorus content of unknowns over a period of one hour with an error of less than 0.2 mgm. % P.

Acknowledgment

The author wishes to take this opportunity to thank Dr. J. B. Collip and Dr. J. C. Paterson for their encouragement of this work.

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THE EFFECT OF IODOACETATE ON THE APPARENT SYNTHESIS OF DIPHOSPHOTHIAMINE DURING THE ESTIMATION OF THE COCARBOXYLASE ACTIVITY OF BLOOD EXTRACT[1]

BY M. C. BLANCHAER AND L. H. COHEN

Abstract

The usual method of determining diphosphothiamine (DPT) by its cocarboxylase activity proved unreliable when applied to blood extract because of a marked upward drift of the results during the period of measurement. This phenomenon, which was associated with excessively high recovery values, was tentatively attributed to a synthesis of DPT since the presence of 1×10^{-3} molar iodoacetate abolished the increase in carboxylase activity. Judging from recovery experiments, the addition of iodoacetate to the reactants permitted a more reliable estimation of the DPT content of blood extract.

Introduction

Diphosphothiamine (DPT) in blood and tissues is commonly estimated by its cocarboxylase activity in the decarboxylation of pyruvate by yeast.

$$CH_3COCOOH \xrightarrow[\text{Yeast carboxylase}]{\text{DPT}} CH_3CHO + CO_2$$

Addition of tissue extracts to this system is reported to result in the synthesis of small amounts of DPT from thiamine and phosphate (2). However, the quantities formed are apparently insufficient to interfere with the determination of DPT in muscle, liver, kidney, and brain (3, 4). The present report deals with a similar effect occurring in the presence of blood extract and with its influence on the estimation of DPT in blood.

Methods

1. *Preparation of Blood Extract*

Blood extract was prepared as described by Westenbrink *et al.* (7). Two ml. of fresh defibrinated human blood was added to an equal volume of 0.28 *N* hydrochloric acid and heated with continuous stirring for one and one-quarter minutes in a boiling water bath. Upon removal from the bath 0.5 ml. of 6.5% potassium hydroxide was added with vigorous stirring. The preparation was immediately centrifuged for 15 min. at 2500 r.p.m. in order to clear the supernatant blood extract. In recovery experiments, 0.25 volumes of a 10 or 20 μgm. % DPT solution was added to one volume of the extract. An equal volume of water replaced the DPT solution in the corresponding control estimations.

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[1] *Manuscript received June 15, 1949.*

Contribution from the Nutrition Laboratory, Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Man.

2. *Preparation of Yeast Carboxylase*

Dried brewer's yeast was washed with alkaline phosphate buffer just before use to remove preformed DPT. The procedure was as follows: 0.6 gm. of powdered yeast was suspended, with stirring, in 50 ml. of 0.1 *M* phosphate buffer, pH 9.0, and the supernatant was discarded after centrifuging. This process was repeated with the same buffer, again with water, and finally with 0.1 *M* phosphate buffer pH 6.2. Exactly four minutes was allowed for suspension of the yeast in the various solutions and three minutes for each centrifugation at 2500 r.p.m.

The washed yeast was resuspended for use in 6.0 ml. of 0.1 *M* phosphate buffer, pH 6.2, containing 1.0 mgm. each of magnesium and manganese ions as the chloride salts and 1.0 mgm. of thiamine chloride. Thiamine, magnesium, and manganese serve as activators of yeast carboxylase (3).

Since calcium interferes with the estimation (6), all water used in washing the yeast and the preparation of reagents was redistilled in glass over potassium carbonate. The distillate was discarded until neutral to methyl red.

3. *Determination of Cocarboxylase Activity*

The cocarboxylase activity of blood extract was estimated by its ability to stimulate the decarboxylation of pyruvate (7). Carbon dioxide produced in the reaction was measured manometrically (5) in 15 ml. Warburg flasks at 27.5° C. in an atmosphere of air. The activity of standard amounts of DPT (0.00, 0.05, 0.10, and 0.20 μgm. in 2 ml. of water) was determined simultaneously.

The following were placed in the main compartment of the Warburg flasks: 0.2 ml. of 2 molal phosphate buffer, pH 6.2; 2.0 ml. of blood extract or standard DPT solution; and 0.5 ml. of the final yeast suspension. In certain experiments indicated below, sufficient sodium iodoacetate was dissolved in the 2 molal buffer to bring the contents of the flasks to a final iodoacetate concentration of 1×10^{-3} molar. The side arm of the flasks contained 5 mgm. of sodium pyruvate in 0.1 ml. of 0.1 *M* phosphate buffer pH 6.2.

The flasks, attached to manometers, were equilibrated for 10 min. in the bath. The pyruvate was then tipped in and the shaking continued. At the end of five minutes, zero readings were taken and the taps closed. Carbon dioxide production was measured at varying intervals for periods up to 70 min. At each time interval the apparent DPT content of the blood extract was read directly from the corresponding standard curve (Fig. 1). In analyzing the results, variation from the mean was expressed as the standard deviation.

Results

In the flasks containing DPT dissolved in water, the rate of carbon dioxide production remained constant for approximately 50 to 60 min. following a short initial lag period. The total volume of gas released at any given time was usually proportional to the DPT content of the flasks over the range

0.00 to 0.20 μgm. DPT (Fig. 1). In the vessels containing blood extract, decarboxylation followed a similar course for a short time and then showed a gradual acceleration that became more marked as time elapsed (Fig. 2A). Table I shows the effect of this phenomenon on the apparent DPT content of an extract prepared from another sample of blood. The upward trend of the results is representative of similar changes in 18 additional experiments in which the average values increased 15% ± 7 between the 20 and 40 min. readings. This drift was accentuated by the addition of DPT to the extract as shown in Table I. In 16 additional recovery experiments cocarboxylase activity increased 24% ± 8 between the 20 and 40 min. readings, resulting in recovery values that rose from 119% ± 19 to 159% ± 20.

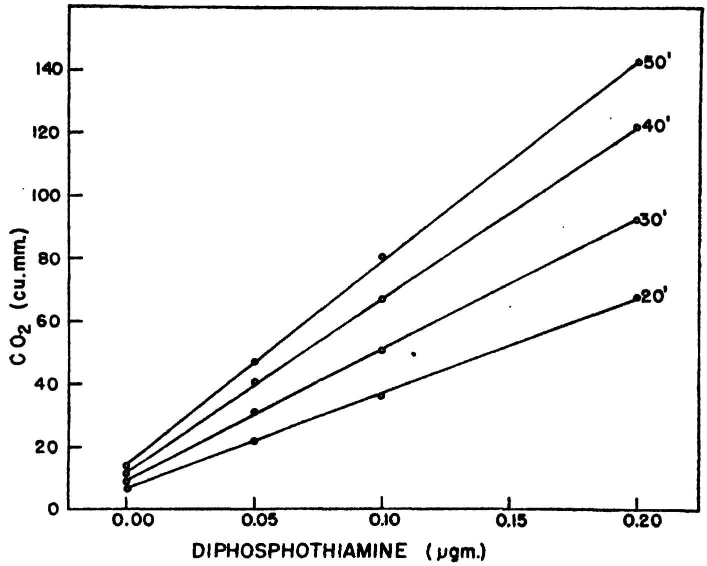

FIG. 1. *Typical standard curves of carbon dioxide production at 20, 30, 40, and 50 min.*

TABLE I

APPARENT DPT CONTENT OF BLOOD EXTRACT (μgm.) AT VARIOUS TIMES

Time (min.)	Blood extract			Blood extract + 0.04 μgm. DPT			Mean recovery* (%)
	Flask 1	Flask 2	Mean (1)	Flask 3	Flask 4	Mean (2)	
10	0.078	0.069	0.074	0.100	0.126	0.113	98
15	0.077	0.068	0.073	0.113	0.137	0.125	130
25	0.087	0.081	0.084	0.144	0.148	0.146	155
30	0.090	0.087	0.088	0.161	0.177	0.169	202
35	0.097	0.097	0.097	0.177	0.196	0.187	225

* *Recovery (%)* $= \frac{mean\ (2) - mean\ (1)}{0.04} \times 100.$

Since the method as described was obviously unreliable, it was felt that a study of the more probable explanations for the upward drift might form a basis for improving the procedure. Goodhart and Sinclair (1) reported a similar increase in the values, which they ascribed to a decrease in carbon dioxide production in the standard flasks due to a drop in pH after the first 30 min. In the present study, adequate buffering maintained the initial pH of 6.2 for at least one hour. The nature of the gas production curves (Fig. 2A) also indicated that the effect was due to an increased activity in the presence of blood extract and not to a decreased rate of decarboxylation in the standard flasks.

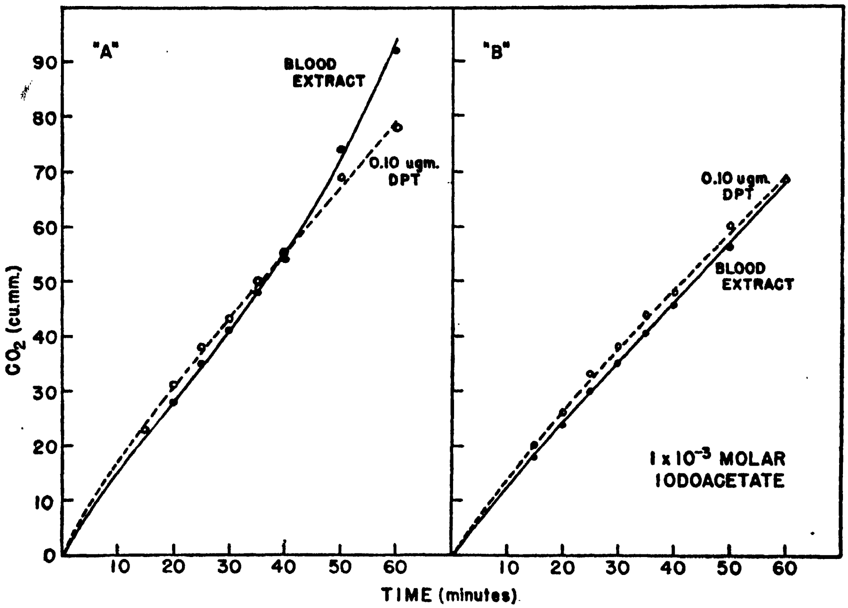

FIG. 2. *A comparison of the rate of carbon dioxide production in the presence of blood extract and 0.1 µgm. DPT, with and without iodoacetate.*

The possibility of DPT being synthesized during the course of the estimation was next examined. Unfortunately, a direct investigation of changes in the DPT content of the extract during the decarboxylation reaction was impossible because of the lack of reliable independent methods for estimating this substance. However, indirect evidence was obtained by adding to the system iodoacetate, which is stated to inhibit the synthesis of DPT (2). The results shown in Fig. 2A and B were obtained simultaneously using the same blood extract. It will be seen that decarboxylation in the presence of 1×10^{-3} molar iodoacetate was slightly depressed. However, gas production in the vessel containing blood extract followed an almost linear course similar to that in the standard flask. The inhibitory effect of iodoacetate on the drift of the values with time is evident when the results in Table II are compared with those in Table I. In eight additional determinations using iodoacetate

TABLE II

APPARENT DPT CONTENT OF BLOOD EXTRACT (μgm.) AT VARIOUS TIMES IN THE PRESENCE OF 1×10^{-3} MOLAR IODOACETATE

Time (min.)	Blood extract			Blood extract + 0.08 μgm. DPT			Mean recovery* (%)
	Flask 1	Flask 2	Mean (1)	Flask 3	Flask 4	Mean (2)	
20	0.070	0.080	0.075	0.153	0.166	0.160	106
25	0.070	0.079	0.075	0.157	0.166	0.162	107
30	0.067	0.076	0.071	0.153	0.161	0.157	107
40	0.067	0.073	0.070	0.157	0.160	0.158	110
50	0.068	0.072	0.070	0.153	0.160	0.157	109
60	0.068	0.077	0.072	0.152	0.157	0.155	102
70	0.063	0.075	0.069	0.151	0.156	0.154	106

* *Recovery* $(\%) = \dfrac{\textit{mean}\ (2) - \textit{mean}\ (1)}{0.08} \times 100.$

the 40 min. values decreased 1% ± 5.3 from the 20 min. readings. Over the same period, the mean recovery of DPT in seven experiments increased from 105% ± 7.4 to 107% ± 10.4.

Discussion

The results reported here show that iodoacetate abolishes the increase in carboxylase activity that occurs with time. Judging from the recovery experiments, the use of iodoacetate permits a more reliable estimation of the DPT content of blood extract. Further work is required to establish whether the rise in carboxylase activity inhibited by iodoacetate truly represents a synthesis of DPT.

Since workers (1, 7) using previous methods for estimating DPT in blood have failed to describe an upward drift of their results, it seems possible that our difficulties arose from the use of a strain of yeast with an unusually great ability to synthesize DPT. For this reason, the results presented above do not necessarily invalidate previously published methods for estimating DPT in blood. Nevertheless, the usual practice of terminating such determinations 30 min. after the addition of pyruvate limits the potential sensitivity and accuracy of the estimations because of the large percentage error involved in measuring the small volumes of gas produced during this short initial period.

The present study indicates that the presence of iodoacetate in appropriate concentrations largely overcomes the objections to the prolonging of the determinations for an additional 30 min., thereby permitting an increasing degree of sensitivity and accuracy in DPT estimations on blood extract.

Acknowledgments

This work was supported by a grant from the National Research Council, Canada, to the senior author (M.C.B.), a Markle Scholar. Merck and Co., Rahway, kindly supplied liberal amounts of diphosphothiamine. The authors wish to thank Profs. J. Doupe and R. J. Rossiter for criticism and advice. We are indebted to J. Crust and Pamela Weiss for technical assistance.

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LIPOTROPIC ACTIVITY OF CHOLINE, BETAINE, AND METHIONINE IN DUCKLINGS[1]

BY R. BERNARD AND J. M. DEMERS

Abstract

A purified diet, deficient in choline and containing 18% casein, results in fatty infiltration of the liver in ducklings killed at four weeks. Total liver lipids is about 13%, the normal value being 6 to 7%. Addition of 0.1 or 0.3% choline chloride results in nearly normal livers. In this respect, ducklings differ from chicks and are comparable to rats and dogs. Betaine has a slight lipotropic activity but does not stimulate growth and has no antiperotic properties. DL-methionine, when added to a purified diet containing 9% casein or to a simplified diet containing 15% casein, has no lipotropic activity. On the contrary, this amino acid increases the total lipids and the severity of fatty infiltration of the livers. Choline has a triple function in ducklings; it promotes growth, prevents hepatic fatty infiltration, and perosis.

Introduction

It is now well established that choline controls fat deposition in the liver of mammals such as rats, dogs, and pigs (5, 4, 8). In rats fed choline deficient diets, the livers are enlarged and pale in color. Microscopically, fat droplets are seen in the hepatic cells. The droplets are frequently large enough to push the cytoplasm and nucleus aside. Moreover, Griffith and Wade (10) found that, if young rats are used, a severe renal hemorrhagic degeneration occurs. The addition of 0.1% choline to a deficient diet prevents fatty livers and kidney damage in rats. In this animal, betaine is also effective and its activity has been found to be about one-third that of choline (25). Methionine also protects young rats against hemorrhagic degeneration of the kidney (11) and its lipotropic activity is calculated as one-fifth that of choline (7). The feeding of a diet containing 30% casein supplies enough methionine to obviate the necessity of a choline supplement (24).

Among birds, the effect of choline in promoting growth and preventing perosis has been studied in chicks and young turkeys (15, 17) as well as in ducklings (26, 3). Perosis is characterized by a shortening and thickening of the tarsus and tibia of young birds. Usually a distortion and dislocation of the hock joint results in the slipping of the tendo calcaneus.

Chicks and turkey poults, when fed purified diets, appear to be unable to utilize betaine or methionine as substitutes for choline. However, when simplified diets that contain peanut meal and yellow cornmeal are fed, these supplements promote growth and have antiperotic properties (14, 16, 21, 22).

In rats, methionine and betaine are effective substitutes for choline. Both substances form choline in the body by methylation of ethanolamine as shown by du Vigneaud and Stetten (30, 31). According to Jukes *et al.* (18) such a

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[1] *Manuscript received May 16, 1949.*

Contribution from the Department of Biology, Laval University, Quebec, Que., with the financial assistance of the National Research Council of Canada and the Conseil des Recherches Agricoles, Department of Agriculture, Province of Quebec.

reaction does not occur in the chick. Almquist (2) has set forth the contrast between mammalian and avian nutrition in respect to interrelations between choline, betaine, and methionine.

The lipotropic activity of choline in bird nutrition has received the attention of a few workers. Kilborn (19) found that adult ducks and pigeons fail to show increased liver fat on a choline-free, high-fat diet and in this way they differ radically from mammals (rats and dogs). Shen (27) has shown that force feeding in ducks produces a marked increase in liver fat, and Flock *et al.* (9) have shown the same to be true of geese. Abbott and De Masters (1), on the basis of analyses of seven samples only, claim that choline decreases the percentage of fatty acids in the liver of chickens killed when 290 days old. Hegsted *et al.* (13) found that choline has no lipotropic action on the livers of chicks killed when four weeks old. It thus appeared from these results that the main functions of choline in avian nutrition are limited to the prevention of perosis and the stimulation of growth.

In previous work on the nutrition of ducklings, the authors (3) noticed that choline deficient birds presented large yellow livers apparently rich in fat. Frozen sections of the livers of the deficient birds revealed intense fatty infiltration and it was decided to study at length the lipotropic activity of choline, betaine, and methionine in this avian species.

Material and Methods

Day-old white Pekin ducklings were used in these studies. Upon receipt from the hatchery, they were placed in heated batteries equipped with raised-screen bottoms. Six ducklings were located in each compartment, measuring about 35 in. square. The birds used in experiments involving purified diets were fed a commercial duck feed for two days prior to the experimental period. Food and water were allowed ad libitum. Each group consisted of six ducklings and the experimental periods lasted three or four weeks. The birds were then killed and samples of liver were obtained for chemical and histological analyses. Liver fat (total lipids) was determined on fresh samples according to the procedure of Shipley *et al.* (28). Pieces of liver fixed in 10% formalin were sectioned with the freezing microtome and stained with Sudan IV and hematein.

The composition of the basal diets used is given in Table I. Diet R-49 is patterned after that used by Hegsted (12) in his studies on duck nutrition. The low-casein diet R-53 is similar to those generally used to produce methionine deficiency in the rat. The simplified (semipurified) diet R-2 resembles that used by Jukes (14) in his early work on perosis. The three basal diets differ principally by their casein contents but resemble each other in being deficient in choline.

Three series of experiments were carried out. In the first series, the lipotropic activity of choline and betaine was investigated, using diet R-49, which contains 18% casein. The second series involved the study of the

TABLE I

COMPOSITION OF BASAL DIETS USED IN THE STUDY OF THE LIPOTROPIC ACTIVITY OF CHOLINE BETAINE, AND METHIONINE IN DUCKLINGS

Ingredients	Basal diet number		
	R-49	R-53	R-2
	Composition		
	%		
Main ingredients			
Sucrose	47		
Cornstarch		70	
Corn meal, yellow			65
Casein, Labco	18	9	10
Skim milk, dry			15
Gelatin	10		
Corn oil	10	10	
Soybean oil			3
Fish oil blend[*]	2	2	1
Alfalfa meal			1
Salts IV[**]	5	5	5
$CaH_4(PO_4)_2 2H_2O$	1		
Liver fraction L[†]	4		
Ruffex	3	3	
	Mgm. per 100 gm. of diet		
Vitamins			
Thiamine hydrochloride	0.4	0.4	0.4
Riboflavin	0.8	0.8	0.8
Calcium pantothenate	2.5	2.5	1.5
Nicotinic acid	4.0	4.0	3.0
Pyridoxine hydrochloride	0.4	0.4	0.4
p-Aminobenzoic acid	2.0	2.0	
Biotin	0.02	0.02	
Inositol	100.0	100.0	
2-Methyl naphthoquinone	0.1	0.1	0.1
α-Tocopherol	10.0	10.0	10.0
Pteroylglutamic acid		0.1	

[*] *Vadol Type No. IV, 3000 A, 400 D (A.O.A.C. chick units). Blended fish oils fortified with vitamin* D_3.

[**] *Hegsted, D. M., Mills R. C., Elvehjem, C. A., and Hart, E. B. J. Biol. Chem. 138 : 459. 1941.*

[†] *A source of the unknown vitamins of the B-complex. Supplied by Wilson Laboratories, Chicago, Ill.*

lipotropic activity of methionine and choline with the low-methionine diet R-53 containing only 9% casein. Finally, the lipotropic activity of methionine and choline was studied in ducklings fed a semipurified diet containing approximately 15% casein, which is intermediate between the two levels mentioned above.

Results and Discussion

A. Effect of Choline and Betaine Using Basal Diet R-49, Containing 18% Casein

The results of this first series of experiments are presented in Fig. 1 and Table II. The growth curves show that at four weeks of age, ducklings fed basal diet R-49 weighed about one-third as much as normal individuals fed a commercial duck feed containing more than 24% protein. The supplementation with 0.5% betaine has no effect on growth, while choline at levels of 0.1 or 0.3% resulted in a nearly normal growth. In Table II are found the observations on perosis, fatty infiltration, and total lipids. All ducklings fed the

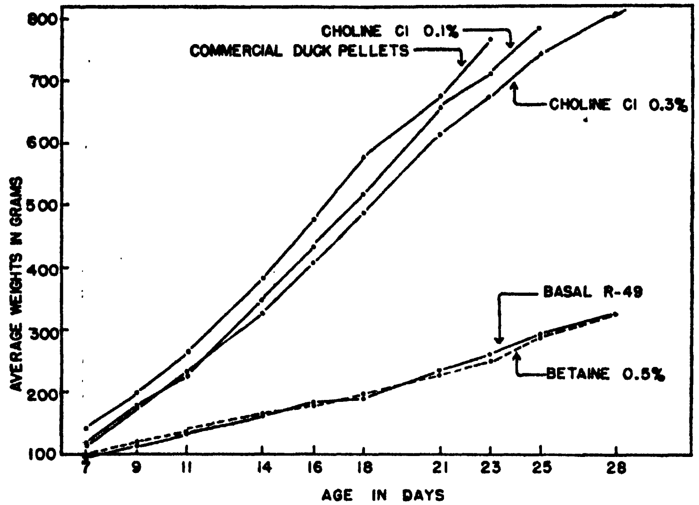

FIG. 1. *Growth curves of ducklings fed purified choline deficient basal diet R-49 (18% casein) only or the same diet supplemented with choline or betaine as compared with commercial duck pellets.*

TABLE II

EFFECT OF SOME LIPOTROPIC FACTORS WHEN ADDED TO AN 18% CASEIN, PURIFIED DIET

Group No.*	Supplement to basal diet R-49	Number with perosis	F.I.**	Liver lipids, %	t††
81	None	6	5	14.4 ± 1.46†	
82	Choline chloride 0.1%	1	2	8.0 ± 1.29	3.26
83	Choline chloride 0.3%	0	3	8.0 ± 0.36	4.27
84	Betaine 0.5%	6	6	10.8 ± 1.95	1.77
80	Commercial duck pellets only	0	0	6.0	

* *Six birds in each group.*

** *Number with fatty infiltration.*

† *Standard error* $= \sqrt{\left(\frac{\Sigma d^2}{n(n-1)}\right)}$.

†† $t > 2.30$ *significant by comparison with control group.*

basal diet showed clear-cut signs of perosis. At a level of 0.3%, choline prevented the malformation entirely. Since one perotic individual was noted in the group supplemented at the 0.1% level, it appears that this particular level is too low for complete protection against perosis but may be satisfactory for optimum growth.

The average lipid content of livers of ducklings fed the commercial diet was 6% and no stainable fat could be detected. On the other hand, the livers of all birds fed the choline deficient diet showed fatty infiltration (Fig. 5*a*) and averaged 14.4% total lipids. The livers of the animals in this last group were pale yellow, soft, and some of them were enlarged. The addition of 0.1 or 0.3% choline chloride resulted in a significant reduction in the liver lipids and the fatty infiltration. However, the percentage of total lipids was not brought entirely back to normal and a few individuals showed some fatty infiltration though it was of a very slight degree. This may indicate that a diet containing 18% casein supplemented with 0.3% choline chloride is still deficient in lipotropic factors. Beveridge *et al.* (6), working with rats, suspect that in addition to methionine, tyrosine may have some lipotropic properties.

These results indicate that in ducklings, choline fulfils three functions: it promotes growth, prevents perosis, and has a definite lipotropic activity. This last function has not been observed in the chick.

Betaine at the level of 0.5% does not improve growth and has no antiperotic properties but rather appears to enhance the symptoms of perosis. There is some indication that this compound has lipotropic activity though the results are not statistically significant. The livers of this group of ducklings were normal in size and color, i.e., dark brown. Microscopic examination revealed a small amount of diffuse fat as compared with the well defined droplets observed in livers of the basal group. It is probable that betaine may favor the synthesis of a limited amount of a cholinelike substance that is used up only for fat transportation and is ineffective in preventing perosis or promoting growth. The results with betaine also suggest that in ducklings as in chicks, the choline requirement may be divided into two parts which McKittrick (23) calls the essential choline and replaceable choline. In ducks, the lipotropic function seems to be the replaceable one. More work on this question is, however, necessary.

B. Effect of Choline and Methionine Using a Purified Diet, R-53, Containing 9% Casein

The results of the second series of experiments are presented in Table III. In all cases the growth was very poor, being on the average about one-fifth that of normal, even when both supplements were added to the basal diet. This is no doubt due to the fact, as was suggested by Knowlton Hall and Sydenstricker (20), that such a diet is known to be suboptimal for many essential amino acids. No signs of perosis were observed, even in the basal

TABLE III

EFFECT OF SOME LIPOTROPIC FACTORS WHEN ADDED TO A 9% CASEIN, PURIFIED DIET

Group No.*	Supplement to basal diet R-53	F.I.**	Liver lipids, %	t	Average weights, gm.	
					4 days	21 days
85†	None	6	34.3 ± 7.48		79	146
86†	DL-methionine 0.5%	6	47.7 ± 2.39	1.70	76	192
87	None	6	29.7 ± 7.06		94	175
88	DL-methionine 0.5%	6	29.8 ± 5.12		86	132
89	Choline chloride 0.3%	4	17.5 ± 5.22	1.39	78	122
90	DL-methionine 0.5%, choline chloride 0.3%	6	14.8 ± 2.69	1.98	70	151

* *Six birds in each group.*

** *Number with fatty infiltration.*

† *Groups 85 and 86 started one month earlier.*

groups. This is probably related to the poor growth rate as has been shown in chicks.

The lipotropic activity of methionine was first investigated by comparing Groups 85 and 86. Much to our surprise, the addition of this amino acid appeared to increase the total lipids of the livers, although the difference is not quite significant. These findings could not be duplicated for Groups 87 and 88, which showed the same average percentage in liver lipids. The livers of ducks fed basal diet R-53 alone or with added methionine were large, pale yellow, or creamy white. Results with Groups 86 and 88 show that, in ducklings, methionine is unable to replace choline as a lipotropic factor. Added evidence was obtained on the lipotropic activity of choline in Groups 89 and 90. In this last group, it is believed that methionine when added with choline may help in decreasing the liver lipids.

In this series of experiments with the low-casein diet, the fatty metamorphosis of livers was most marked in groups in which the diets were supplemented with methionine (Fig. 5*b*). Great individual variations in liver lipids was noted in all groups and this is perhaps related to a multiple deficiency in essential amino acids.

C. Effect of Choline and Methionine Using a Simplified Basal Diet, R-2, Low in Choline and Containing Approximately 15% Casein

In order to gain more information on the importance of methionine, a third series of experiments was conducted using a simplified basal diet (R-2) containing approximately 15% casein. It was thus hoped to avoid multiple amino acids deficiencies and yet still have a low-methionine diet.

The growth curves corresponding to the treatments studied in this series are shown in Fig. 2. The addition of methionine to the basal diet R-2 did not improve growth. On the contrary, there seems to be a slight inhibition. This is supported by the fact that when methionine and choline are added together, the growth is inferior to that when choline alone is added. Perosis was observed in the basal group (Figs. 3 and 4) and in that supplemented with methionine, while the two groups receiving 0.3% choline chloride, with or without added methionine, were entirely protected. It thus appears that methionine is unable to replace choline for this function in this simplified diet.

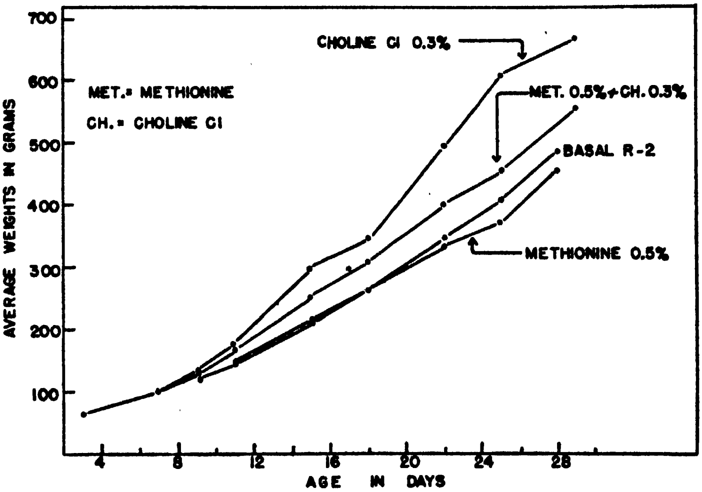

FIG. 2. *Growth curves of ducklings fed simplified choline-low basal diet R-2 (15% casein) only or the same diet supplemented with choline or methionine or both.*

TABLE IV

EFFECT OF SOME LIPOTROPIC FACTORS WHEN ADDED TO A 15% CASEIN, SEMIPURIFIED DIET

Group No.*	Supplement to basal diet R-2	Number with perosis	F. I.**	Liver lipids, %	*t*
91	None	3	6	16.7 ± 1.37	
92	Choline chloride 0.3%	0	6	13.2 ± 2.11	1.41
93	DL-methionine 0.5%	3	6	21.8 ± 2.64	1.70
94	DL-methionine 0.5%, choline chloride, 0.3%	0	6	10.4 ± 1.50	3.12

* *Six birds in each group.*
** *Number with fatty infiltration.*

The livers of ducklings fed the basal diet R-2 contained on the average 16.7% fat. Choline showed a very slight lipotropic effect, while methionine increased the liver lipids to 21.7%. This confirms the results obtained with the 9% casein diet, in that methionine in the experimental conditions outlined is antilipotropic. When both supplements are added (Group 94), there is a significant decrease in liver lipids to 10%, although this is still 4% above normal.

Fatty infiltration was noticed in all the birds of this series of experiments. It was most pronounced in Group 93, supplemented with methionine, and the least in Group 94, which received both supplements. Choline alone had a definite lipotropic action as shown by the comparison of Figs. 5*c* and 5*d*. It is evident that the basal diet R-2 containing 15% casein even when supplemented with methionine and choline is still deficient in lipotropic factors and this probably can be corrected by a higher percentage of casein. Looking back to the first series of experiments, it will be seen that, when an 18% casein basal diet is used, choline alone brought the liver lipids practically to normal.

From the results presented in this work, it is suggested that there exists a marked difference between chick and duckling in so far as fat accumulation and transport in the liver is concerned.

Acknowledgments

The authors wish to thank Ayerst, McKenna, and Harrison for supply of Blended Fish Oils; Merck and Co., for biotin and α-tocopherol; Hoffmann-La Roche for biotin, α-tocopherol, riboflavin, pantothenic acid, thiamine hydrochloride, and pyridoxine hydrochloride; and Lederle Laboratories for pteroylglutamic acid (Folvite).

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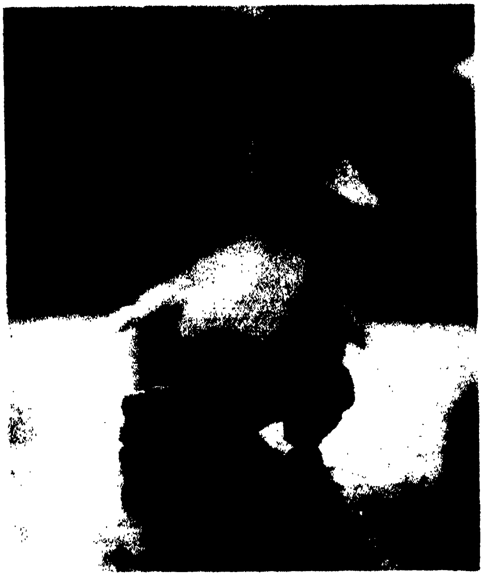

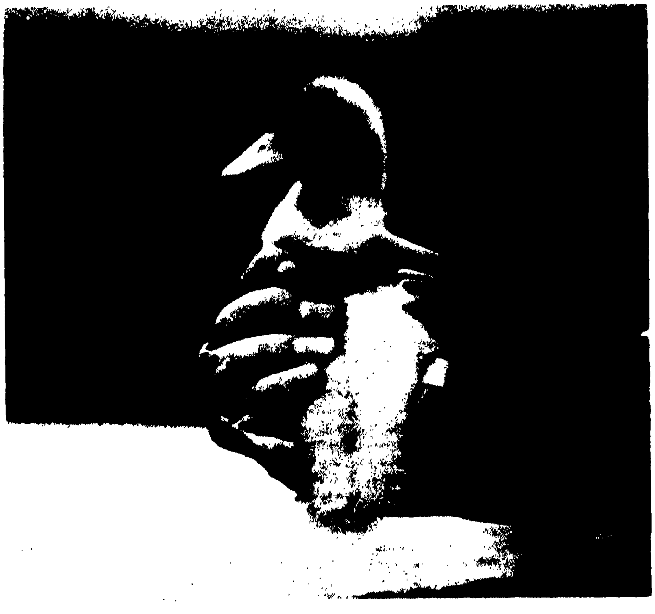

FIG. 3. *Perotic duck. Age: 16 days. Diet R-2 low in choline. Note toes curled in.*

FIG. 4. *Same animal as above. The flattening of the tibiometatarsal joints and deviation of the right leg are well shown.*

PLATE II

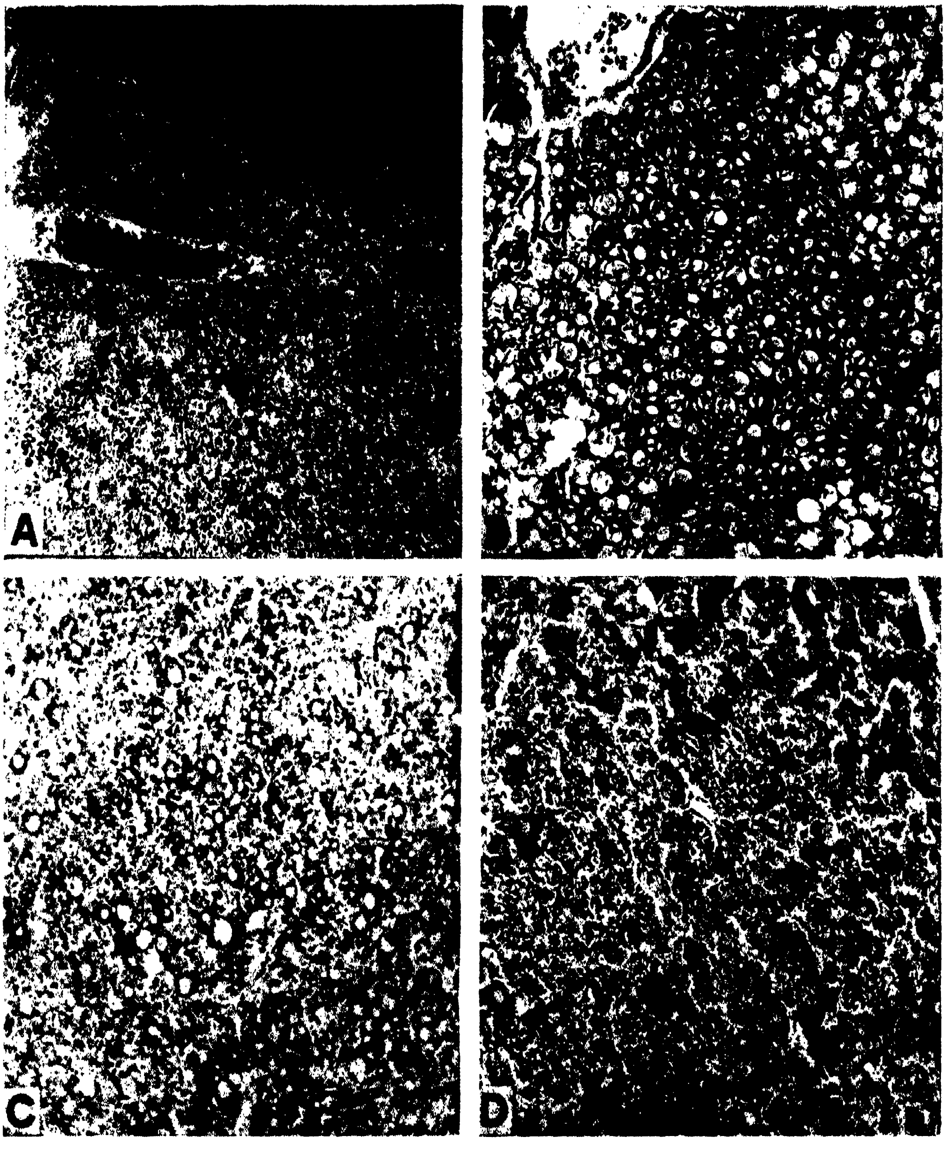

FIG. 5. *Photomicrographs of liver sections of ducklings. Frozen section stained with Sudan IV and hematein. Approx.* × *113.*

A. Purified basal diet R-49 (18% casein), choline deficient. The fat droplets are small and uniformly distributed.

B. Purified basal diet R-53, choline-free and low in casein (9%) supplemented with 0.5% methionine. Note the large amount of fat infiltration.

C. Simplified diet R-2 (15% casein) low in choline. Fatty infiltration is extensive.

D. Diet R-2 plus 0.3% choline chloride. The fatty infiltration is reduced but still evident.

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PHOSPHATASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES[1]

By D. M. Cram and R. J. Rossiter

Abstract

Rabbit polymorphonuclear leucocytes contain an active phosphatase that readily hydrolyzes disodium phenyl phosphate. The pH activity curve of the enzyme was found to have two maxima, one in the region of pH 10 and the other in the region of pH 5. The alkaline phosphatase was much more active than the acid phosphatase. The concentration of alkaline phosphatase in rabbit white cells was approximately one thousand times that of the enzyme in the serum. Under the conditions of study, the alkaline phosphatase activity was proportional to the concentration of the enzyme. The effect of substrate concentration on the enzyme activity was studied and the Michaelis constant (K_s) determined. An excess of substrate inhibited the enzyme. The course of the reaction was linear with time for the first 60 min.; after 90 min. the activity fell off faster than would be expected if the reaction were of the first order.

Magnesium and glycine, in low concentrations, caused an increase in the enzyme activity, whereas zinc, cyanide, borate, phosphate, bile salts, and glycine, in higher concentrations, were inhibitory. Fluoride had no demonstrable effect. Surface-active substances, such as saponin, bile salts, or alkyl sulphate, liberated the enzyme from the cells. Similar results were obtained when α-glycerophosphate or β-glycerophosphate was used as the substrate.

The alkaline phosphatase can be considered to belong to Class AI of Folley and Kay (22) and the acid phosphatase to Class AII. The alkaline phosphatase can also be considered to be a Phosphatase II of Cloetens (9).

Introduction

Phosphatases, or perhaps more correctly, phosphomonoesterases, have been found in a great many tissues. References to most of the work on these enzymes are given in the excellent reviews of Kay (39), Folley and Kay (22), and Moog (47).

Kay (37, 38) suggested that white cells may contain a phosphatase and, subsequently, Roche (53, 54) demonstrated alkaline phosphatase activity in mixed white cell preparations containing both granulocytes and lymphocytes, obtained from the blood of the rabbit, guinea pig, or horse. Umeno (60) measured the phosphatase activity of white cells from the peritoneal exudate of a rabbit; no mention was made of the type of cell present or of the pH of the reaction mixture. More convincing, however, were the experiments of Fiessinger and Boyer (16) on the alkaline phosphatase of rabbit white cells. Both alkaline and acid phosphatase have also been demonstrated in bone marrow (27, 65).

Although these reports indicate beyond doubt that there is an alkaline phosphatase in white cells, they are quantitatively misleading. It has recently been shown (Rossiter (56)) that surface-active substances such as saponin, bile salts, or alkyl sulphate liberate large quantities of alkaline phosphatase from rabbit polymorphonuclear leucocytes into the surrounding

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[1] *Manuscript received April 19, 1949.*

Contribution from the Department of Biochemistry, University of Western Ontario. The research was supported by a grant from the National Research Council of Canada.

fluid. This phosphatase would not be measured if, as in previous experiments on white cell phosphatase, estimations were done on suspensions of intact cells. The difficulties of extracting enzymes from cells are very real, and, for phosphatases, the problem has been adequately discussed (22, 39). By using saponin to extract the enzyme from the leucocytes, some of the difficulties have been overcome. A preliminary account of these experiments has already appeared (Cram and Rossiter (11)).

Methods

Cell Preparations

Polymorphonuclear leucocytes were obtained from the rabbit by the method of de Haan (28). Warm sterile isotonic saline (300 to 500 ml.) was run into the peritoneal cavity and drained off three to four hours later into a beaker containing a few grains of heparin. Alternatively, the saline was left in the animal over night. A further quantity of saline was run in the following morning and withdrawn immediately. In each instance the resulting opalescent fluid contained 2000 to 16,000 leucocytes per cu. mm., at least 95% of which were polymorphonuclear.

Phosphatase Determination

Phosphatase was usually determined by the method of King and Armstrong (41), in which the phenol, liberated by the hydrolysis of disodium phenyl phosphate, is measured by the method of Folin and Ciocalteu (18). To a centrifuge tube was added:

2 ml. buffer (either acetate buffer pH 4.9 or carbonate–bicarbonate buffer pH 9.9),
2 ml. 0.01 *M* disodium phenyl phosphate,
0.5 ml. 1% saponin,
0.3 ml. 0.017 *M* magnesium chloride.

The tube was then placed in a 37° C. water bath and, after temperature equilibration, 0.2 ml. of the cell suspension was added. The cells of 0.2 ml. suspension were thus extracted by fluid containing 5 mgm. saponin or the cells of 1 ml. by fluid containing 25 mgm. saponin. This amount of saponin has previously been shown to be optimal (56). Exactly one hour after the addition of the cell suspension, 1.8 ml. Folin and Ciocalteu reagent (diluted 1 : 3) was added and, after mixing, the tube was centrifuged. The phenol in 4 ml. of the supernatant was then determined as described by King and Armstrong (41). The test was always run in duplicate. Control tubes were also set up in which all the substances of the test, except the cell suspension, were incubated for one hour. The Folin and Ciocalteu reagent was then added, followed by the cell suspension. The color of the experimental tube was read against that of the control tube in a Coleman Universal Spectrophotometer at 650 mμ.

In experiments with either α-glycerophosphate or β-glycerophosphate as substrate the inorganic phosphorus liberated by the enzyme was measured by the method of Fiske and Subbarow (17) after the proteins had been precipitated with 3 ml. 20% trichloroacetic acid. The color was read in a Coleman Universal Spectrophotometer at 650 mμ.

Recording of Results

Usually the results were expressed as the amount of phenol or phosphorus (in mgm.) liberated by 100 ml. enzyme preparation in one hour. In the section on the quantitative determination of the enzyme in the leucocytes, the results were given in King–Armstrong (41) units, as subsequently modified (43), per 10^{10} cells. These results were also brought into line with other estimates of the concentration of enzymes in white cells by converting them into King–Armstrong units per mgm. acid-insoluble phosphorus, using the relation that 10^{10} cells contain 6.8 mgm. acid-insoluble phosphorus previously established for the rabbit polymorphonuclear leucocyte (Rossiter (57)).

Results

Concentration of Phosphatase in Rabbit Polymorphonuclear Leucocytes

The concentration of alkaline (pH 9.9) and acid (pH 4.9) phosphatase in rabbit polymorphonuclear leucocytes is given in Table I. The mean concentration of alkaline phosphatase was 207 (±68) King–Armstrong units per 10^{10} cells or 31 (±10) King–Armstrong units per mgm. acid-insoluble phosphorus. This corresponds approximately to 52 King–Armstrong units per gm. fresh tissue or 320 King–Armstrong units per gm. dry tissue. The concentration of acid phosphatase was much less (7.8 ± 3.7 King–Armstrong units per 10^{10} cells).

TABLE I

PHOSPHATASE ACTIVITY OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Cell count W.B.C./cu. mm.	Alkaline phosphatase activity			Acid phosphatase activity		
	Mgm. phenol/100 ml. enzyme/hr.	King–Armstrong units/10^{10} cells	King–Armstrong units/mgm. acid-insol. phosphorus	Mgm. phenol/100 ml. enzyme/hr.	King–Armstrong units/10^{10} cells	King–Armstrong units/mgm. acid-insol. phosphorus
3000	42	343	50	1.2	10	1.5
3300	33	245	36	0.9	7	1.0
4100	30	189	28	2.1	13	1.9
7400	36	121	18	2.7	10	1.5
4000	18	113	17	0.9	1	0.2
6900	60	209	31	1.1	4	0.6
4200	36	216	32	1.0	6	0.9
1800	15	216	32	0.8	11	1.6
	Mean (±S.d.)	207 (± 68)	31 (± 10)	Mean (±S.d.)	7.8 (± 3.7)	1.1 (± 0.5)

Other Substrates

In the experiments reported in Table I disodium phenyl phosphate was the substrate. Table II shows that, in addition to phenyl phosphate, the enzyme was also able to hydrolyze either α-glycerophosphate or β-glycerophosphate. In each instance the substrate concentration was optimal. The enzyme hydrolyzed phenyl phosphate more rapidly than either of the glycerophosphates, and β-glycerophosphate more rapidly than α-glycerophosphate. This has also been observed for the alkaline phosphatases of other tissues (12, 35, 40, 42, 51). The optimum pH for each of these substrates is slightly different. In this respect the work of King and Delory (42) is important, for they found that the rate of hydrolysis of these three substrates was in the same order, even if measurements were made at the optimum pH for each.

TABLE II

HYDROLYSIS OF DIFFERENT SUBSTRATES BY RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Substrate	Concentration	Phosphatase activity (mgm. phosphorus/100 ml. enzyme/hr.)		
		Preparation *A* (cell suspension)	Preparation *B* (saponin supernatant)	Preparation *C* (cell suspension)
Disodium phenyl phosphate	0.004 *M*	2.76	5.78	19.7
Sodium α-glycerophosphate	0.02 *M*	0.68	1.80	9.6
Sodium β-glycerophosphate	0.02 *M*	0.87	2.11	10.0

Enzyme Concentration

Fig. 1 shows that, under the conditions of the study, the extent of the hydrolysis of phenyl phosphate was proportional to the concentration of the enzyme.

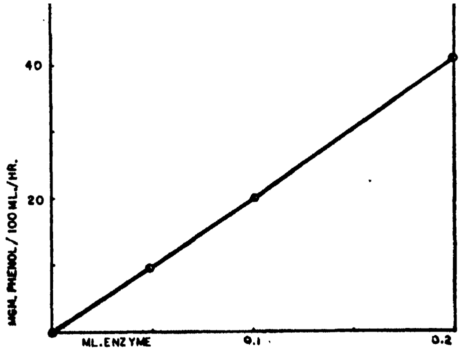

FIG. 1. *The relation between enzyme activity and concentration of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M. Incubation time, 1 hr. Temperature, 37° C.*

Substrate Concentration

The effect of substrate concentration on the fully activated enzyme system is shown in Fig. 2, which gives the results of a typical experiment. The optimum substrate concentration was in the region of 0.004 *M*, concentrations greater than this being inhibitory. The Michaelis constant, K_s, for six different enzyme preparations with enzyme concentrations extending over a sixfold range, was evaluated by the method of Lineweaver and Burk (44). The figures varied from 0.00059 to 0.00096. Fig. 2 shows that in a typical experiment the Lineweaver and Burk (44) plot of *1/S* against *1/V* was reasonably linear. The pH was 9.9. These results can be compared with those of Folley and Kay (21) who found a value of 0.0006 for mammary gland alkaline phosphatase at pH 10. Schmidt and Thannhauser (58), using a highly purified intestinal alkaline phosphatase, found that the K_s for phenyl phosphate was 0.000072 and 0.000078 for two different concentrations of the enzyme. However the study was made at pH 9.3, which is not the optimum for phenyl phosphate. Folley and Kay (21) found that the K_s of mammary gland phosphatase was 0.0001 instead of 0.0006 when measurements were made at pH 8.9 rather than pH 10, while Jacobsen (33) reported that the K_s of alkaline phosphatase was greatly affected by changes in pH. It thus appears probable that, had Schmidt and Thannhauser (58) determined the K_s at pH 10, they would have obtained a higher figure.

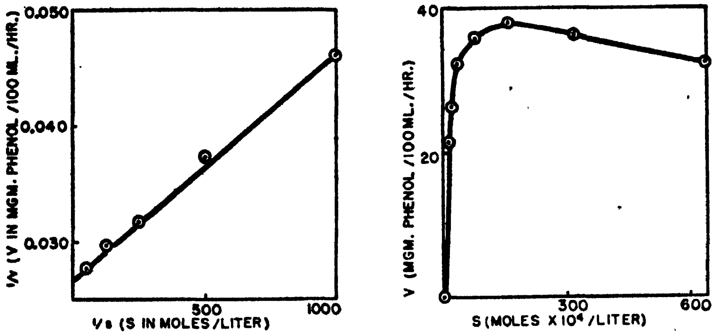

FIG. 2. *The effect of substrate concentration on the activity of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate. Temperature, 37°C.*

Martland and Robison (45), studying the hydrolysis of glycerophosphate by bone phosphatase, found no difference in enzyme activity when the substrate concentration ranged from 0.3 *M* to 0.003 *M*, a result confirmed by other workers when glycerophosphate was the substrate (8, 32, 58). Fig. 2 shows that this was not so for rabbit polymorphonuclear leucocyte phosphatase with phenyl phosphate as substrate; excess substrate was inhibitory, a result confirmed by Folley and Kay (21) for mammary gland phosphatase and Schmidt and Thannhauser (58) for intestinal phosphatase.

Haldane (30) suggested that the inhibition of an enzyme by excess of the substrate was due to one or more molecules of the substrate combining with the dissociable enzyme–substrate complex to form a further addition compound, which does not break down to give the products of the reaction. Thus we have

$$E + S \rightleftharpoons ES \text{ (active)}$$
$$ES + S \rightleftharpoons ES_2 \text{ (inactive)}$$

where ES dissociates into the products of the reaction and ES_2 is inactive and does not dissociate. The constant of the first reaction is K_s and that of the second can be represented by K_2. A mathematical development of this theory gives

$$\frac{S}{V} = \frac{K_s}{V_M} + \frac{1}{V_M}\left(S + \frac{S}{K_2}\right)$$

where S is the concentration of the substrate, V_M is the maximum velocity and V the observed initial velocity of the hydrolysis (Lineweaver and Burk (44), Case III).

In one experiment, in which higher substrate concentrations than those of the experiment of Fig. 2 were used, K_2 was evaluated for the rabbit polymorphonuclear leucocyte alkaline phosphatase. The mean value was 0.5, but the inaccuracies of the procedure were such that it was impossible to test the validity of the Haldane theory in this instance.

Course of Reaction with Time

A number of experiments using different enzyme preparations, enzyme concentrations, or substrate concentrations showed that the course of the reaction was linear with time for the 60 min. incubation period investigated. This meant that, under the conditions of study, the amount of phenol liberated in one hour gave an adequate measure of the initial velocity of the reaction.

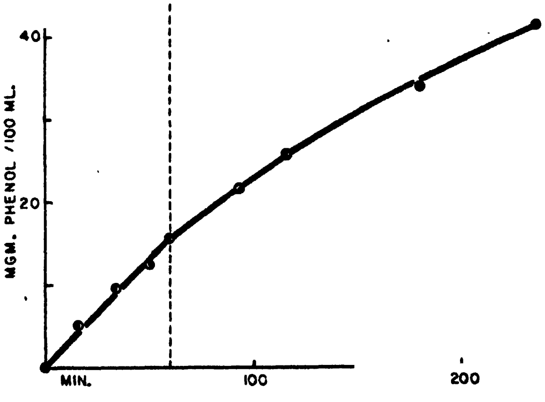

FIG. 3. *Time course of reaction for rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M. Temperature, 37° C.*

The same was true for other alkaline phosphatases (21, 32, 58). Fig. 3 represents the results of one of three experiments designed to show the effect of incubation periods longer than one hour. The results of the other experi-

TABLE III

COURSE OF REACTION WITH TIME. ENZYME, RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. TEMPERATURE, 37° C.

	Time (hr.)	Phenol formed (mgm.)	Percentage hydrolysis	k $\left(\frac{2.303}{T} \log \frac{S}{S-X}\right)$
Experiment 1 (Saponin supernatant)	1	0.0299	1.59	0.0161
	2	0.0535	2.84	0.0146
	3	0.0695	3.71	0.0127
	4	0.0822	4.37	0.0112
Experiment 2 (Cell suspension)	1	0.0587	3.12	0.0320
	2	0.1105	5.88	0.0304
	3	0.1465	7.80	0.0273
	4	0.1745	9.28	0.0246

ments are given in Table III, which shows the amount of phenol liberated, the percentage hydrolysis of the substrate, and the first-order reaction constant calculated from:

$$k = \frac{2.303}{T} \log \frac{S}{S - X}$$

where S is the concentration of the substrate and X is the amount of substrate hydrolyzed in T hours. In each instance, k became less as the reaction proceeded. Since the substrate concentration was optimal, one would not expect the course of the hydrolysis of phenyl phosphate to simulate that of a first-order reaction. First order kinetics are usually not observed for enzymatic reactions until the substrate concentration has fallen considerably below the level necessary to "saturate" the enzyme. Table III shows that the rate of hydrolysis of phenyl phosphate fell off more rapidly than would be expected if the reaction were described in terms of the unimolecular law. This means that some explanation, other than substrate depletion, must be sought to explain the decrease in activity. It has been suggested (13, 45) that this progressive decrease in activity might be caused by the inhibitory effect of inorganic phosphate formed during the course of the reaction.

Phosphate Inhibition

Table IV shows that 0.01 *M* phosphate greatly inhibited the enzyme and that concentrations as low as 0.0003 *M* caused an observable inhibition. This would favor the suggestion (45) that the falling off in activity faster than would be expected by the unimolecular law was due to phosphate inhibition. If, as in Experiment 2, Table III, hydrolysis had proceeded to the extent of 10% of the substrate, the phosphate concentration would be 0.0004 *M*, a concentration in excess of 0.0003 *M*, which was definitely inhibitory (Table IV).

The effect of phosphate on other alkaline phosphatases has also been described (45, 53, 58). By far the most extensive study was that of Jacobsen

TABLE IV

EFFECT OF PHOSPHATE ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Phosphate concentration	Preparation 1 (cell suspension)		Preparation 2 (cell suspension)		Preparation 3 (alkyl sulphate supernatant)	
	Mgm. phenol /100 ml. /hr.	Relative activity in presence of PO^4, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of PO_4, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of PO_4, %
0	2.8	100	17.7	100	25.8	100
0.00031 *M*	2.4	86	15.6	88		
0.00062 *M*	2.5	89	15.9	90		
0.00125 *M*	2.1	75	15.0	85		
0.0025 *M*	2.0	72	13.2	75		
0.005 *M*	1.8	64	10.5	59		
0.010 *M*	1.4	50	8.1	46	12.7	49

(32), who showed that the inhibition of kidney alkaline phosphatase by phosphate was partly competitive and partly noncompetitive, i.e. independent of substrate concentration.

Hydrogen Ion Concentration

Many of the earlier workers on alkaline phosphatase reported that the optimum pH was in the region of 9 (35, 36, 38, 52, 53). It is now known that the optimum pH of alkaline phosphatase depends upon both the nature (42) and the concentration (21, 33) of the substrate. Also true initial velocities must be measured; that this has not always been done may be the reason for many of the discrepancies found in the literature.

Fig. 4 shows the pH activity curve of rabbit polymorphonuclear phosphatase using the "universal buffer" of Prideaux and Ward (49). The optimum is at pH 10. Using borate, diethylbarbiturate, or glycine buffers, the optimum pH was, in each instance, between 9.75 and 10.0. This agrees with the results of Folley and Kay (21), who found the optimum pH of mammary gland phosphatase to be 10 with 0.0045 *M* phenyl phosphate as substrate, and King and Delory (42), who found the optimum pH of intestinal phosphatase with 0.002 *M* phenyl phosphate to be 9.76.

Many of the buffers commonly used for determining the effect of pH on enzyme systems inhibit the alkaline phosphatase of rabbit polymorphonuclear leucocytes. Table V shows the extent of this inhibition with each of the four buffers used. In each instance the pH was adjusted to 9.9 before the test and the concentration of buffer was the same as that used in determining the optimum pH of the enzyme. That diethylbarbiturate inhibited bone alkaline phosphatase considerably less than the same concentration of glycine has been reported previously (2). For all subsequent work on the effect of pH on the enzyme activity, a modification of the Michaelis (46) diethylbarbiturate buffer, described by King and Delory (42), was used.

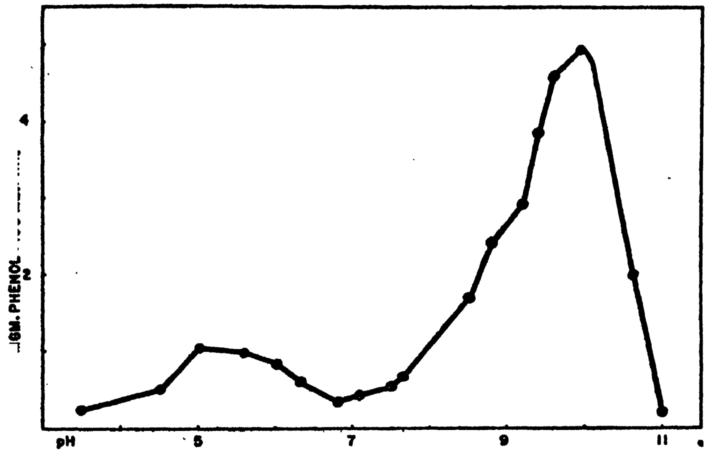

FIG. 4. *The effect of hydrogen ion concentration on the activity of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M. Incubation time, 1 hr. Temperature, 37° C.*

TABLE V

EFFECT OF VARIOUS BUFFERS ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Buffer	Phosphatase activity (mgm. phenol/100 ml./hr.)		Relative activity in presence of additional buffer, %
	No additional buffer	Additional buffer	
Borate	9.6	6.8	71
Universal buffer	10.2	5.6	55
Diethyl-barbiturate	7.4	6.9	93
Glycine	21.1	8.8	42

Fig. 5 shows the effect of substrate concentration on the pH optimum. With lower substrate concentrations the optimum pH was lower, a result reported for other alkaline phosphatases (21, 33).

The pH activity curve of the phosphatases from many tissues has two maxima, one in the neighborhood of 5 and one between 9 and 10 (3, 4, 12, 48). It is now generally conceded that there are at least two different phosphatases in most tissues and Perlmann and Ferry (48) have succeeded in separating the phosphatase of kidney into two fractions, one active at low and one at high pH values. The rabbit polymorphonuclear leucocyte phosphatase, with maxima in the region of pH 5 and pH 10, is thus similar to the phosphatases of other tissues.

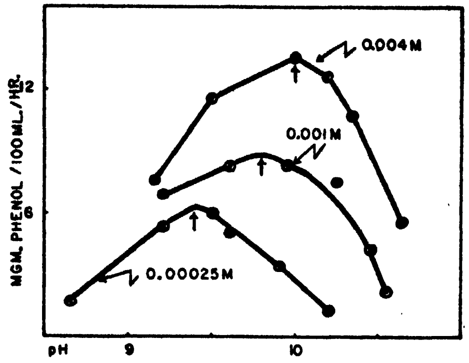

FIG. 5. *The effect of substrate concentration on the optimum hydrogen ion concentration of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate. Incubation time, 1 hr. Temperature, 37° C.*

Magnesium

The activity of the rabbit polymorphonuclear leucocyte phosphatase was increased greatly in the presence of magnesium ions. Table VI shows that the mean activity in the presence of 0.001 *M* magnesium was approximately three times that of the unactivated enzyme. The optimum concentration of magnesium was 0.001 *M* (Fig. 6). This effect of magnesium ions on alkaline phosphatase activity is well known and will be discussed in greater detail in a subsequent paper.

TABLE VI

EFFECT OF MAGNESIUM IONS ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 hr. TEMPERATURE, 37° C.

Phosphatase activity (mgm. phenol/100 ml./hr.)		Relative activity in presence 0.001 *M* Mg^{++}, %
No Mg^{++}	0.001 *M* Mg^{++}	
53.4	210.0	392
13.2	44.1	330
10.7	18.3	175
10.5	22.8	217
9.3	30.1	323
9.0	26.6	295
9.0	24.6	274
5.8	17.2	296
3.8	11.9	313
3.6	11.7	321
2.9	5.8	200
2.3	9.1	391
1.3	3.9	300
	Mean (± S.d.) =	302.1 (± 63.0)

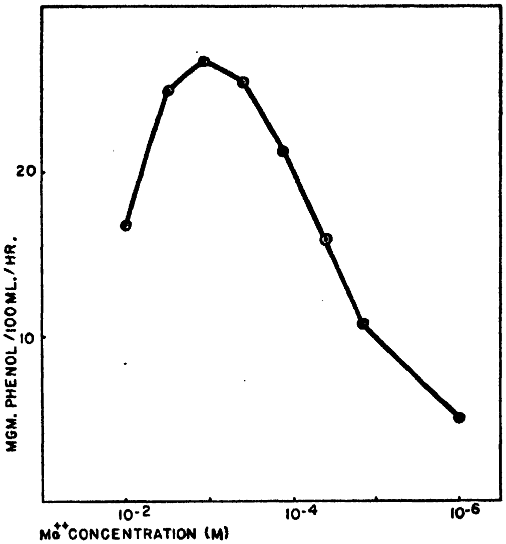

FIG. 6. *The effect of magnesium ions on the activity of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M. Incubation time, 1 hr. Temperature, 37°C.*

Surface-active Substances

It was reported by Rossiter (56) and Cram and Rossiter (11) that surface-active substances, such as saponin, alkyl sulphate,* or bile salts, liberated alkaline phosphatase from the rabbit polymorphonuclear leucocyte. Table VII shows the effect of these substances in concentrations that were found to be optimal. The effect of sodium taurocholate was less than that of saponin or alkyl sulphate for, as will be shown in the following section, this concentration of bile salt was itself inhibitory to cell-free preparations of the enzyme.

TABLE VII

EFFECT OF SURFACE-ACTIVE SUBSTANCES ON THE LIBERATION OF ALKALINE PHOSPHATASE FROM SUSPENSIONS OF WASHED RABBIT POLYMORPHONUCLEAR LEUCOCYTES. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. MAGNESIUM CONC., 0.001 *M*. INCUBATION TIME, 1 HR. TEMPERATURE 37° C.

Cells suspended in:	Alkaline phosphatase activity (mgm. phenol/100 ml./hr.)	
	Suspension *A*	Suspension *B*
Isotonic saline	5.7	16.8
1% Saponin in isotonic saline	12.3	57.1
0.0625% Alkyl sulphate in isotonic saline	12.4	58.8
0.00625 *M* sodium taurocholate in isotonic saline	10.6	50.0

"Purified alkyl sulfate" provided by the Procter and Gamble Company, Cincinnati, Ohio.

Inhibitors

Bile salts, glycine, zinc, or cyanide were found to inhibit the alkaline phosphatase of the rabbit polymorphonuclear leucocyte, while fluoride was without effect.

Bile Salts

Whereas bile salts increased the activity of suspensions of cells by liberating the enzyme into the surrounding fluid, the effect on cell-free preparations was invariably inhibitory (Table VIII). Bodansky (7) found that bone, kidney, or serum alkaline phosphatase was reduced to 65 to 75% of its activity in the presence of 0.00125 *M* sodium desoxycholate and to 45 to 55% of its activity in the presence of 0.00625 *M* sodium taurocholate while intestinal phosphatase was not inhibited. This result has recently been confirmed (55). The white cell enzyme was inhibited to 80 to 90% of its activity in the presence of 0.00125 *M* sodium desoxycholate and to 65% of its activity in the presence of 0.00625 *M* sodium taurocholate, thus resembling the phosphatase of bone, kidney, or serum rather than that of the intestinal mucosa.

TABLE VIII

EFFECT OF BILE SALTS ON CELL-FREE PREPARATIONS OF RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 M. INCUBATION TIME, 1 HR. TEMPERATURE 37°C. 1% SAPONIN SUPERNATANT

Bile salt	Concentration	Phosphatase activity (mgm. phenol/100 ml. enzyme/hr.)		Relative activity in presence of bile salt, %
		No bile salt	Bile salt	
Sodium desoxycholate	0.00125 *M*	27.3	24.2	89
Sodium desoxycholate	0.00125 *M*	21.6	17.4	81
Sodium taurocholate	0.00125 *M*	27.3	23.4	86
Sodium taurocholate	0.00125 *M*	21.6	18.9	87
Sodium taurocholate	0.00125 *M*	60.0	51.0	85
Sodium taurocholate	0.00625 *M*	60.0	38.7	65

Glycine

Table IX shows that in the presence of low concentrations of glycine (optimum 0.005 *M*) the activity of the enzyme was increased, but that in the presence of high concentrations of glycine the enzyme was inhibited. This activating effect of low concentrations of glycine has frequently been reported for the phosphatases of other tissues (5, 6, 62), but Schmidt and Thannhauser (58) found that 0.0015 *M* glycine had no effect on a purified intestinal phosphatase. The inhibitory action of high concentrations of glycine is also well known (5, 6, 8, 62).

TABLE IX

EFFECT OF GLYCINE ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Glycine concentration	Preparation 1 (cell suspension)		Preparation 2 (cell suspension)	
	Mgm. phenol /100 ml./hr.	Relative activity in presence of glycine, %	Mgm. phenol /100 ml./hr.	Relative activity in presence of glycine, %
0	5.7	100	13.1	100
0.00007 *M*	6.0	105	12.6	96
0.00021 *M*	5.9	103	12.5	95
0.00062 *M*	7.1	124	14.5	111
0.00186 *M*	7.7	135	17.6	134
0.00557 *M*	8.0	140	19.5	149
0.0167 *M*	8.0	140	18.3	140
0.05 *M*	7.4	130	18.3	140
	Preparation 3 (saponin supernatant)		Preparation 4 (saponin supernatant)	
	Mgm. phenol /100 ml./hr.	Relative activity in presence of glycine, %	Mgm. phenol /100 ml./hr.	Relative activity in presence of glycine, %
0	27.3	100	21.6	100
0.001 *M*	27.5	101	24.3	112
0.01 *M*	24.3	89	21.0	97
0.1 *M*	8.0	29	9.0	42

Zinc

Very low concentrations of zinc ion (10^{-6} *M*) inhibited the enzyme (Table X). Other alkaline phosphatases are progressively inhibited by increasing concentrations of zinc ions (14, 31, 48).

Cyanide

Table XI shows that low concentrations of cyanide (0.0001 *M* and 0.001 *M*) increased the activity of the enzyme, but that concentrations of 0.01 *M* were greatly inhibitory. Cloetens (9, 10) on the basis of cyanide inhibition, described two alkaline phosphatases: Phosphatase I, which was inactive in the absence of magnesium ions and not inhibited by 0.01 *M* cyanide, and Phosphatase II, which was affected variously by magnesium and inhibited by 0.01 *M* cyanide. By measuring the phosphatase activity in the presence of optimal concentrations of magnesium ions and in the presence or absence of 0.01 *M* cyanide, he was able to determine the ratio of Phosphatase I to Phosphatase II in various tissues. For intestine, serum, or bone the ratio was 0 to 14%; for kidney it was 8 to 50% and for liver it was 16 to 90%.

TABLE X

Effect of zinc on rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M. Incubation time, 1 hr. Temperature 37° C.

Zinc concentration	Preparation 1 (cell suspension)		Preparation 2 (cell suspension)		Preparation 3 (saponin supernatant)	
	Mgm. phenol /100 ml. /hr.	Relative activity in presence of Zn^{++}, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of Zn^{++}, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of Zn^{++}, %
0	15.3	100	7.1	100	60.0	100
10^{-6} M	10.7	70	6.1	85	60.0	100
10^{-5} M	5.3	34	3.4	48	30.9	52
10^{-4} M	3.6	23	2.8	39	5.7	11
10^{-3} M	3.2	21	2.0	28	4.2	7

TABLE XI

Effect of cyanide on rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M or sodium β-glycerophosphate 0.02 M. Incubation time, 1 hr. Temperature, 37° C.

Cyanide concentration	Preparation 1 (cell suspension)		Preparation 2 (cell suspension)		Preparation 3 (cell suspension)	
	Mgm. phenol /100 ml. /hr.	Relative activity in presence of cyanide, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of cyanide, %	Mgm. phosphorus /100 ml. /hr.	Relative activity in presence of cyanide, %
0	9.0	100	8.0	100	1.2	100
10^{-4} M	12.2	135	10.7	133	2.0	167
10^{-3} M	11.7	129	11.1	138	1.6	133
10^{-2} M	1.8	20	1.2	15	0.15	13

Table XII shows that the rabbit polymorphonuclear leucocyte alkaline phosphatase was greatly inhibited by 0.01 M cyanide so that, in this respect, it resembled the phosphatase of intestine, serum, or bone rather than that of liver.

Fluoride

Low concentrations (0.0001 M) of fluoride ion had no effect on the enzyme, but 0.01 M fluoride increased the activity of the enzyme slightly (Table XIII). Although the alkaline phosphatase of kidney is not inhibited by fluoride, the phosphatase of yeast, active at pH 6.2, is greatly inhibited (1). In addition, Cloetens (10) found that Phosphatase I was inhibited in the presence of 0.01 M fluoride, but that Phosphatase II was not affected. Thus, on a basis of fluoride insensitivity as well as of cyanide sensitivity, the alkaline phosphatase of the

TABLE XII

EFFECT OF CYANIDE ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 M OR SODIUM GLYCEROPHOSPHATE 0.02 M. MAGNESIUM CONCENTRATION, 0.01 M. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C. FINAL CONCENTRATION OF CYANIDE, 0.01 M

Preparation	Substrate	Phosphatase activity (mgm. phenol (or P.) /100 ml./hr.)		Relative activity in presence of cyanide, %
		No cyanide	Cyanide 0.01 M	
Cell suspension	Disodium phenyl phosphate	9.0	1.8	20
Cell suspension	Disodium phenyl phosphate	8.0	1.2	15
Cell suspension	Sodium β-glycerophosphate	1.2	0.15	13
Cell suspension	Disodium phenyl phosphate	30.6	3.9	12
Saponin supernatant	Disodium phenyl phosphate	26.9	1.4	5
Cell suspension	Sodium α-glycerophosphate	6.9	2.7	39
Cell suspension	Sodium β-glycerophosphate	7.6	2.1	28

rabbit polymorphonuclear leucocyte can be considered to be a Phosphatase II of Cloetens. The enzyme is not the specific hexose diphosphatase described by Gomori (26), since that enzyme is inhibited by fluoride and activated by cyanide.

TABLE XIII

EFFECT OF FLUORIDE ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 M. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Fluoride concentration	Preparation 1		Preparation 2	
	Mgm. phenol /100 ml./hr.	Relative activity in presence of fluoride, %	Mgm. phenol /100 ml./hr.	Relative activity in presence of fluoride, %
0	10.0	100	7.7	100
10^{-4} M	10.0	100	8.0	104
10^{-3} M	10.0	100	7.7	100
10^{-2} M	10.4	104	9.0	117

Discussion

To compare the alkaline phosphatase content of one tissue with that of another is difficult because, in addition to the uncertainties of extraction already mentioned, each worker has usually employed his own methods and has expressed his results in a variety of units. However, the figure of 52 King–Armstrong units (43) per gm. fresh tissue for the rabbit polymorphonuclear leucocyte is of the same order as that reported by Folley and Greenbaum (19) for the alkaline phosphatase of both the kidney and the mammary gland of the rat. Expressed in the units employed in this study, the figures for the rat become 60 units per gm. fresh tissue for kidney and 7.5 units per gm. for resting mammary gland, rising to 30 units per gm. during lactation (20). Thus the concentration of alkaline phosphatase in the rabbit polymorphonuclear leucocyte is greater than that found in most other organs, for it is generally conceded that kidney and lactating mammary gland are two of the tissues of the body richest in alkaline phosphatase (22).

These experiments lend support to the suggestion of Roche (53) that the phosphatase of the white cell might be a source of the alkaline phosphatase of the serum. Fiessinger and Boyer (16), because of the low concentration of phosphatase that they found in white cells, considered this to be unlikely. We now know, however, that the concentration of alkaline phosphatase in the polymorphonuclear leucocyte of the rabbit is some 10 times greater than that reported by Fiessinger and Boyer (16). But the possibility still remains that the white cell may have obtained its phosphatase by adsorption of the enzyme from the plasma. If this were so, the white cells must have the extraordinary ability of concentrating the enzyme by a factor of 1000, for the concentration of alkaline phosphatase in the rabbit polymorphonuclear leucocyte is of the order of 5000 King–Armstrong units per 100 gm. packed white cells compared with 5 units per 100 ml. for rabbit plasma (Haight and Rossiter (29)).

It seems clear that the alkaline phosphatase is within the cell rather than adsorbed on the cell surface, for its presence has been demonstrated by a number of workers (15, 24, 34, 61, 63) using the histochemical techniques of Gomori (23) and Takamatsu (59). Gomori (25) also claimed that there was little acid phosphatase in polymorphonuclear leucocytes, a result that has subsequently been confirmed (50, 63, 64).

The alkaline phosphatase of the polymorphonuclear leucocyte of the rabbit resembles the alkaline phosphatase of bone or kidney in that it has a similar optimum pH, K_s, and sensitivity to inhibitors such as phosphate, high concentrations of glycine, bile salts, zinc ions, cyanide, or fluoride. It also resembles the bone and kidney phosphatase in that its activity is increased by magnesium ions or low concentrations of glycine. It differs from the alkaline phosphatase of the intestine in that it is inhibited by bile salts and differs from the principal alkaline phosphatase of the liver in that it is inhibited by cyanide and not by fluoride. The enzyme can be considered to belong to Class AI of Folley and Kay (22) and the acid phosphatase to Class AII. The alkaline phosphatase can also be classed as a Phosphatase II of Cloetens (9).

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 27, SEC. E. DECEMBER, 1949 NUMBER 6

INTERFACIAL PRECIPITATION OF CHOLESTEROL IN HUMAN AND "ARTIFICIAL" BILES[1]

BY PHILIP FITZ-JAMES AND ALAN C. BURTON

Abstract

Bile was withdrawn from human gall bladders at different levels and analyzed for cholesterol and bile salts. Results showed there was a chemical stratification corresponding to that in specific gravity already demonstrated. Samples of bile were layered with distilled water in plastic cells. Precipitation of cholesterol soon occurred at the interface. Chemical studies showed this was due to a difference in rate of diffusion of cholesterol and bile salts across the interface, leading to a local fall of the critical ratio. Eventually the formation of a pseudo "stone" was observed. The role of interfacial precipitation due to differential diffusion in cholelithiasis is discussed.

Introduction

There has long been radiological evidence (4, 1, 2, 5) suggesting that bile in the gall bladder is not uniform but often stratified in layers of different composition. Campbell and Burton (3) demonstrated such stratification, with respect to specific gravity and color index, in the gall bladders of normal cattle and in some human gall bladders. Floating gall stones were often found to be suspended near the interface between two such strata, suggesting that deposition of cholesterol might be favored at this place.

The experiments here reported were designed first to verify the inference that there was a stratification in concentration of bile salts and of cholesterol, corresponding to the stratification in specific gravity already reported, and secondly to study the physicochemical changes that might occur at interfaces between two such strata.

Methods

Human bile was used in these experiments. Samples of bile removed by Dr. B. A. Campbell from human gall bladders for specific gravity determinations were studied chemically for bile salt (as cholic acid) and for free cholesterol. Bile salt as cholic acid was determined by the Reinhold and Wilson (7) modification of the Myelius reaction. Cholesterol was determined as free cholesterol by a modification of the method of Schoenheimer and Sperry (8). Some of the samples were removed *in situ* at operation, the others were taken at 1 cm. distances from the upright bladder immediately after removal.

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[1] *Manuscript received in original form May 2, 1949, and, as revised, August 8, 1949. Contribution from the Department of Biophysics, University of Western Ontario, London, Ont.*

For the studies of physicochemical changes occurring at interfaces, samples of gall bladder bile were obtained from patients during operation. These were placed in 15 ml. centrifuge tubes and carefully overlaid, without mixing, with distilled water, observed and photographed for changes at the interface. Samples were removed at different levels by a fine pipette after different periods of time for chemical study. Control tubes were prepared using equal quantities of the bile and of distilled water, but these were thoroughly mixed immediately.

In order to clarify the explanation of the phenomena that were observed, similar experiments were then made using an "artificial bile" consisting of cholesterol with bile salts (either cholic or desoxycholic acid). One hundred ml. of a 9% solution of sodium cholate in double distilled water was mixed with a suspension of cholesterol in distilled water, prepared according to the method of Porges and Neubauer (6). The cloudy solution that resulted was adjusted to pH 7.5 with dilute sodium hydroxide and filtered in the cold. A clear solution resulted, which was autoclaved without visible change. This artificial bile was then layered, with sterile technique, under distilled water in lucite cells as follows: 50 ml. of the artificial bile was added to a cell and an equal volume of distilled water placed on top by allowing it to run down the tilted chamber from a fine bore pipette. This was done in duplicate, so that one cell could be used for withdrawing samples, another for photographing the changes. In addition a control cell of smaller dimensions was set up with equal quantities of the artificial bile and water, the only difference being that these were mixed thoroughly instead of being layered. In a second set of experiments a 5% solution of sodium desoxycholate was used instead of the cholate. Desoxycholate was measured by a modification of the Toshio Simada Method (9).

From the cell with rubber-stoppered side holes, samples were carefully drawn off at definite levels with a 22 gauge hypodermic needle. Usually 1.2 ml. was taken from 10 to 12 different levels. On these samples cholate and cholesterol were determined by the above methods. In both sets of experiments samples were taken at 24 hr. and at 70 hr. after layering.

Results

(a) *Existence of a Chemical Stratification*

Table I gives results of chemical analysis on samples taken at different levels in the gall bladders of four patients. These chemical studies show that stratification of bile as found by specific gravity determinations is accompanied by a definite difference in concentration of bile salts and cholesterol. In some of the cases studied, the ratio of cholesterol to cholic acid changed abruptly from level to level. It can be concluded that the stratification of bile in gall bladders, demonstrated by Campbell and Burton (3), involves a "chemical" stratification as well as stratification with respect to specific gravity.

TABLE I

BILE SALTS AND CHOLESTEROL AT DIFFERENT LEVELS IN HUMAN GALL BLADDERS

Case No.	Description of sample	Sample No.	Specific gravity (courtesy of Dr. B. A. Campbell)	Bile salt as cholic acid, mgm. %	Free cholesterol, mgm. %	Ratio cholic acid: cholesterol
1	Samples removed immediately after operation, 1 cm. apart	1	1.0275	1040	151	6.9 : 1
		2	1.040	1500	236	6.3 : 1
		3	1.0475	1930	250	7.7 : 1
		4	1.055	2140	356	6.0 : 1
2	Samples taken *in situ*	A_1, top	1.0125	344	77	4.4 : 1
		A_2, center	1.0250	600	125	4.8 : 1
		A_3, bottom	1.0325	1460	259	5.6 : 1
	Postoperative samples 1 cm. apart	B_1	1.0200	670	138	4.8 : 1
		B_2	1.0200	650	134	4.8 : 1
		B_3	1.0250	935	167	5.6 : 1
		B_4	1.0375	1430	269	5.3 : 1
		B_5	1.0375	1320	300	4.4 : 1
		B_6	1.0375	1560	300	5.2 : 1
3	Postoperative samples 1 cm. apart	1	1.0150	2200	214	10.2 : 1
		2	1.0150	2230	284	7.8 : 1
		3	1.0150	2330	275	8.5 : 1
		4	1.0150	2500	300	8.3 : 1
		5	1.0150	3000	330	9.1 : 1
		6	1.0150	3100	317	9.8 : 1
		7	1.0150	3000	328	9.2 : 1
		8	1.0175	3100	385	7.9 : 1
		9	1.0175	3100	440	7.1 : 1
		10	1.0275	4150	580	7.2 : 1
4	Samples taken *in situ*	1, top bile	1.005	340	63	5.4 : 1
		2, midway	1.010	710	87	8.6 : 1
		3, bottom	1.015	804	102	7.9 : 1

(*b*) *Phenomena Occurring in Human Bile Layered with Distilled Water*

Bile from four different patients, layered with distilled water, was observed. In two days three of the samples showed fine granular or cloudy suspension just above the original bile–water interface, and the fourth showed a heavy interfacial precipitate. In contrast, control tubes in which the same quantities of bile and distilled water had been thoroughly mixed (i.e. no layering) showed no trace of precipitation. The fourth tube and its control were photographed at seven days (Fig. 1) and samples were taken for chemical study after nine days with results as indicated also in Fig. 1. Table II gives the detailed data.

A study of these results indicates that the bile salts had moved up into the distilled water somewhat ahead of the cholesterol. Eventually the diffusing cholesterol was left in an environment so critically low in bile salt (and possibly other hydrophilic substances) that it passed out of solution as shown in the photograph. Possibly the specific gravity of this cholesterol was insufficient to allow complete settling out, although the last picture in the study (Fig. 1) indicates considerable sediment. The control tube of mixed bile and water showed no precipitation.

TABLE II

CONCENTRATIONS AT DIFFERENT LEVELS IN "LAYERED" BILE AFTER NINE DAYS

Sample	Cholic acid, mgm. %	Free cholesterol, mgm. %	Ratio cholic acid: cholesterol
1	49.6	6.2	8.0 : 1
2	81	8.3	9.7 : 1
3	138	12.3	11.2 : 1
4	236	17.6	13.3 : 1
5	300	25.3	11.8 : 1
6, just above cloudy zone	417	48.2	8.7 : 1
7, in cloudy zone	620	153.4	4.1 : 1
8	758	96.9	7.5 : 1
9	990	122	8.1 : 1
Mixed control tube	346	40.4	8.6 : 1

(c) *Phenomena Occurring with "Artificial Bile" Layered with Distilled Water*

Fig. 2 shows the series of changes seen when artificial bile was layered. Within an hour of layering with the cholate, both "sample" and "photographic" cells showed distinct double zones of cloudiness at the bile–water interface. At 18 hr. of layering the cloudy zones were still double in both cells but had moved up the cells slightly. After 24 hr. the clouds were still higher. The upward movement of the cloudy zone continued for the duration of the study period (144 hr.) and two zones of cloudiness were still distinguishable, as illustrated, until 96 hr., after which the clear zone between them disappeared and the cloudiness gradually spread evenly in the upper part of the cell (130 to 144 hr.).

Results with sodium desoxycholate instead of sodium cholate were similar. Within half an hour a fine white cloudy layer was seen at the interface, and this moved slowly upwards, becoming more diffused. The cloudy zone appeared single rather than double (in contrast to cholate).

Observation of the cloud formed with cholate over even longer periods revealed an interesting phenomenon. The cloud on reaching the top of the fluid in the cell gradually shrank to a loose white ball. Over a period of days this aggregation gradually settled, disintegrating as it sank (Fig. 3). Eventually the reduced ball of flocculum settled on the bottom and gradually went back into solution until it was clear, and the ratio of cholesterol to cholic acid was then the same throughout.

Control cells showed no precipitation throughout the experiments and bacteriological studies failed to demonstrate organisms that could be responsible for the cloudiness.

PLATE I

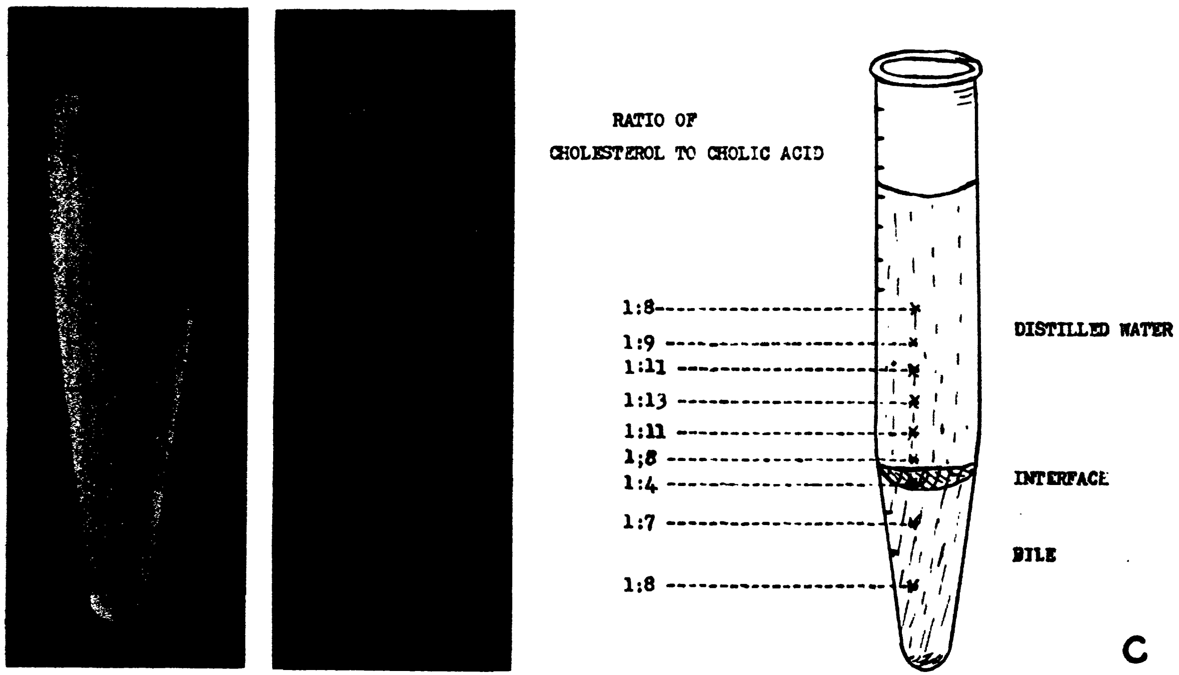

FIG. 1. *Phenomena in bile layered with distilled water. "A" control tube, "B" precipitation (at arrow) in layered tube, "C" results of chemical studies on "B".*

PLATE II

FIGURE 2

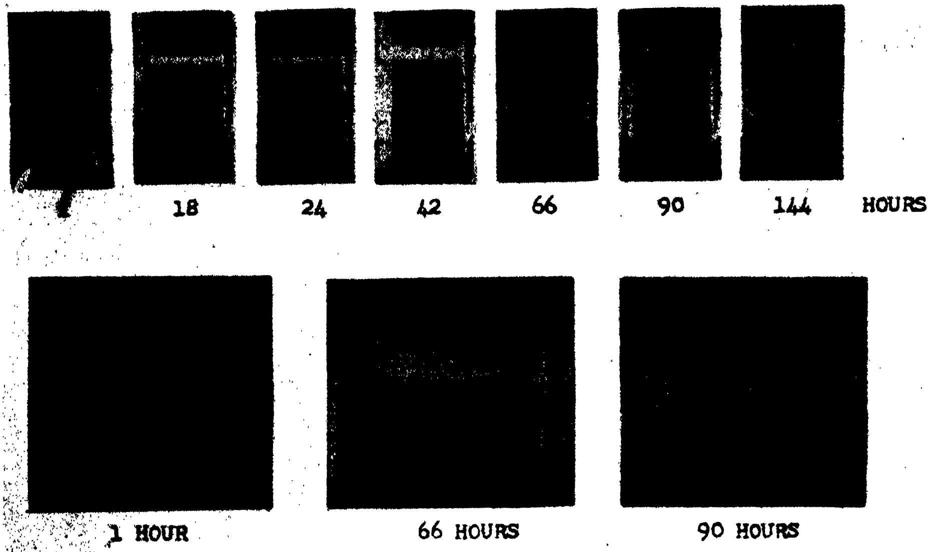

FIG. 2. *Phenomena in sodium cholate – cholesterol solution layered with distilled water.*

FIGURE 3

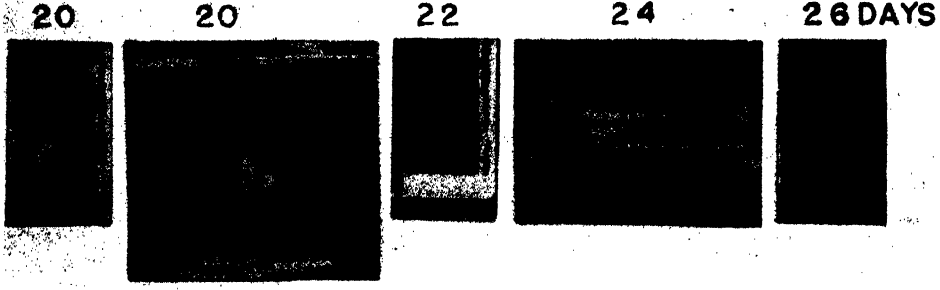

FIG. 3. *Formation of a cholesterol "stone" in layered sodium cholate – cholesterol solution.*

The results of the chemical analysis are shown in the graphs, Fig. 4, when sodium cholate was used, and Fig. 5 with sodium desoxycholate, after 24 hr. and after 70 hr.

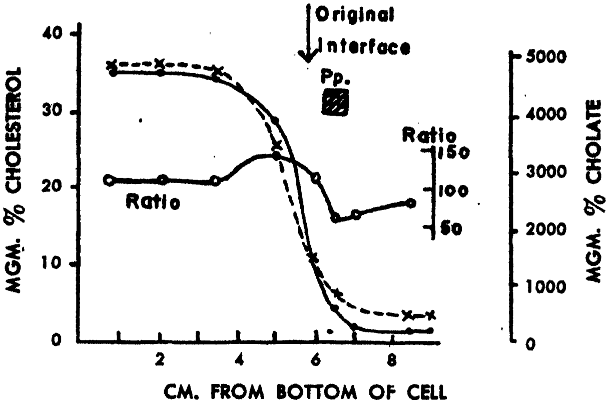

FIG. 4. *Distribution of cholesterol and sodium cholate in cell 24 hr. after layering with distilled water. Pp indicates the zones of cloudy precipitation.* – – – – – *Cholesterol.* ——— *Cholate.*

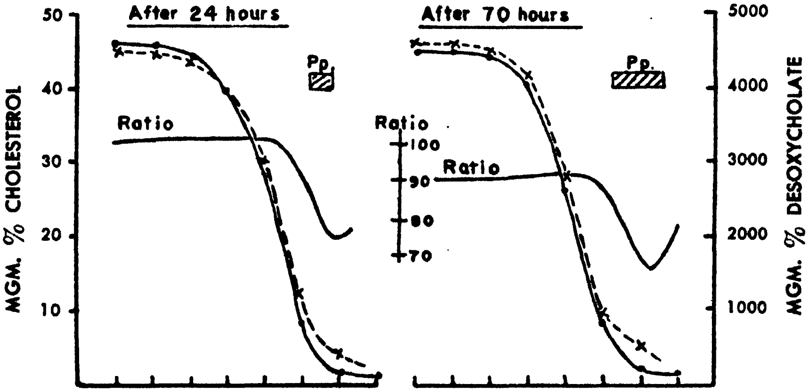

FIG. 5. *Distribution of cholesterol and sodium desoxycholate 24 hr. and 70 hr. after layering with distilled water. Pp indicates the zones of cloudy precipitation.* – – – – *Cholesterol.* ——— *Desoxycholate.*

Discussion

From the graphs (Figs. 4 and 5) it can be seen that cholesterol diffuses upwards across the interface into the distilled water ahead of the bile salts. Thus the ratio of bile salts to cholesterol rises slightly below the interface, and at the same time falls abruptly above the interface. The heaviest zone of precipitate is found slightly below the region where the chemical studies show the minimum ratio of bile salts to cholesterol. This, and the fact that the cloudiness extends below into a region of high ratio, may well be due to a settling of the colloidal particles that have been precipitated.

The steady rise of the top of the cloud is in accordance with the theory of the different rates of diffusion of bile salts and cholesterol, as the region of minimum ratio will rise as diffusion proceeds. The rise of the bottom of the cloud suggests that the precipitated cholesterol is redissolved as it enters a region of higher ratio.

One difference between the results on actual human bile and those cholesterol – bile salt solutions must be noted. In the former it appears that the cholic acid diffuses upward across the interface faster than the cholesterol, whereas the reverse is true with the "artificial bile". The presence in the bile of other substances such as protein or mucoid substances, which may associate with the cholesterol and slow its diffusion, makes this explicable. As long as there is a difference in rate of diffusion of the cholesterol and the substances necessary to keep it in solution, in whichever direction this difference may be, an "interfacial precipitate" may result. The eventual re-solution of the precipitate when diffusion is complete is also in accordance with the theory, for eventually the ratio of bile salts to cholesterol will be the same (equal to that in the original bile) at all levels. Another difference to be noted is the much greater ratio of bile salts (as cholic acid) to cholesterol required in the "artificial biles" (about 70 : 1 for desoxycholate and 100 : 1 for cholate) to that required in the human bile (less than 10 : 1 from Table II). The difference is to be attributed to the other normal hydrophilic substances in bile.

One can only speculate as to the application of these results to the problem of formation of cholesterol stones in the human gall bladder. The existence of stratification in physiological conditions has been clearly established, and in a gall bladder where contraction and expulsion of the contents is infrequent and incomplete, stratification will persist for long periods. The physico-chemical phenomena that have been described would be likely to occur (between concentrated and more dilute biles) and cholelithiasis could be favored. In any case, the phenomena of "interfacial precipitation", due to a differential diffusion at an interface, is of intrinsic interest and may occur in other biological circumstances. For example, it may well be of importance in the deposition of cholesterol on the wall of blood vessels in arteriosclerosis.

Conclusions

(*a*) It is concluded that the stratification of bile in the gall bladder, already demonstrated by specific gravity measurements to exist in physiological as well as pathological conditions, is accompanied by similar stratification with respect to the concentrations of bile salts and of cholesterol.

(*b*) This chemical stratification can lead to the precipitation of cholesterol because the diffusion of cholesterol and of bile salts across the interface between two layers occurs at different rates. A local change in the ratio of bile salts to cholesterol therefore results and if it falls below a critical value precipitation will result.

(*c*) This mechanism may well play an important role in the precipitation of cholesterol stones in a gall bladder in which, due to inactivity or incompleteness of contraction, stratification is long persistent.

Acknowledgments

We are indebted to Dr. A. Bernstein, whose interest in gallstone formation and earlier work on stratification stimulated this research, and to Dr. B. A. Campbell for supplying us with samples of human bile withdrawn from the gall bladder.

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THE PRODUCTION OF ACUTE MASSIVE HEPATIC NECROSIS IN RATS BY DIETARY MEANS[1]

By M. R. Abell and J. M. R. Beveridge

Abstract

Acute hepatic necrosis has been produced in every rat of a group fed a diet low in protein, relatively deficient in the tocopherols, and containing cod liver oil and rancid lard. The evidence suggests that the latter two components have been instrumental in effecting this high incidence of liver necrosis, possibly by means of their well known deleterious action on the tocopherols.

Introduction

Several groups of investigators (2, 7, 9, 16) have reported the production of massive hepatic necrosis in rats by the feeding of diets low in protein and also deficient in the sulphur-containing amino acids, methionine and cystine. Others have utilized somewhat similar rations, the distinguishing feature of which was also thought to be a deficiency in protein and in these amino acids, but they failed to obtain the same results (10, 13, 14).

An examination of the diets used by authors reporting a significant percentage of necrosis revealed several interesting points of similarity that were lacking in the diets of those who failed to produce the liver lesion. All diets reviewed by us that were notably successful in producing necrosis contained cod liver oil as a source of vitamins A and D; the fat component was relatively low in tocopherols, and furthermore, α-tocopherol was not included in the vitamin supplements. These factors were taken into consideration in the formulation of diets for the production of necrosis.

Experimental

Groups of 10 white male rats of the Wistar strain, average weight 140 gm. (122 to 158 gm.) were placed in individual cages with 1/3 in. screen floors and were offered 8 gm. of the test diets per day. The surviving animals were necropsied after 105 days. Tissue slices from all lobes of the livers of these animals and from animals dying during the experiment were fixed in Zenker's formol and the sections were stained with haematoxylin and eosin and with Masson's trichrome stain. Some of the diets that have been utilized are shown in Table I. The H diets were patterned as closely as possible on those described by Himsworth and Glynn (7), except for the inclusion of rancid fat in the H_3 diet.

The sugar vitamin mixtures used in the H and A diets contributed respectively the following amounts of B vitamins per 8 gm. of food: thiamine hydrochloride, 20 μgm. and 40 μgm.; riboflavin, 25 μgm. and 20 μgm.; pyridoxine

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[1] *Manuscript received July 11, 1949.*

Contribution from the Department of Pathological Chemistry, Faculty of Medicine, University of Western Ontario, London, Ont.

TABLE I

COMPOSITION OF DIETS

	H_2	H_3	A_1	A_3
Yeast	18	18	—	—
Casein	—	—	4	4
Cornstarch	27	27	—	—
Sucrose	—	—	76	78
Lard	50	50*	10	10
Celluflour	—	—	2	2
Cod liver oil	1	1	2	—
Cod liver oil concentrate	—	—	—	0.01
Salt mixture	3**	3**	5***	5***
Vitamin mixture	1	1	1	1

* *Slightly rancid lard, produced by irradiation for 2½ hr. with ultraviolet light.*

** *Himsworth (6).*

*** *Beveridge and Lucas (1).*

Yeast—Fleischmann's active dried baker's.

Casein—fat and vitamin free (Merck).

Cod liver oil—Mead's, 3000 IU of vitamin A and 400 IU of vitamin D per gm.

Cod liver oil concentrate—Ayerst, McKenna, and Harrison, 50,000 IU of vitamin D and 200,000 IU of vitamin A per gm.

Sugar vitamin mixtures—explained in text.

hydrochloride, 20 μgm. and 16 μgm.; calcium pantothenate, 100 μgm. and 80 μgm. In addition, A diets supplied nicotinic acid (80 μgm.) and choline chloride (12 mgm.) per 8 gm. diet.

The animals that died of acute massive hepatic necrosis presented consistent pathological findings. The livers were enlarged, soft, friable, with tense smooth capsules and, in most cases, presented a coarsely mottled appearance due to yellowish-gray necrotic and haemorrhagic areas. Some of the livers were less obviously necrotic on gross inspection, merely presenting the appearance of a marked chronic passive congestion.

Microscopically, the acute necrosis is most extensive, involving completely many contiguous lobules and the greater portion of many other lobules (Fig. 1). Throughout the entire section there are only a few uninvolved lobules and in many cases the only viable tissue that remains is comprised of islands of cells situated near the portal tracts. In the early stages of necrosis the liver cells are pale, finely granular, with fairly distinct cell outlines, but show striking nuclear changes, consisting of karyolysis and karyorrhexis. At this stage there is no inflammatory cell infiltration and essentially no haemorrhage. In some sections the necrotic process appears more advanced, the involved areas presenting a picture of extreme disorganization and consisting of pale eosinophilic granular debris with scattered nuclear fragments, a few polymorphonuclear leucocytes and varying amounts of haemorrhage.

Several animals, after an illness of a few days, recovered, and later, at necropsy, showed varying degrees of postnecrotic scarring. Only occasionally, however, was the postnecrotic state recognizable on gross inspection and in these cases the surfaces showed irregular pitted scars and often a portion of a lobe was markedly shrunken and deformed.

The microscopic findings of postnecrotic scarring vary markedly in severity from a diffuse postnecrotic fibrosis (Fig. 2) to small localized scars.

The results of the experiment with respect to the incidence of hepatic lesions are summarized in Table II.

TABLE II

DIETARY HEPATIC LESIONS

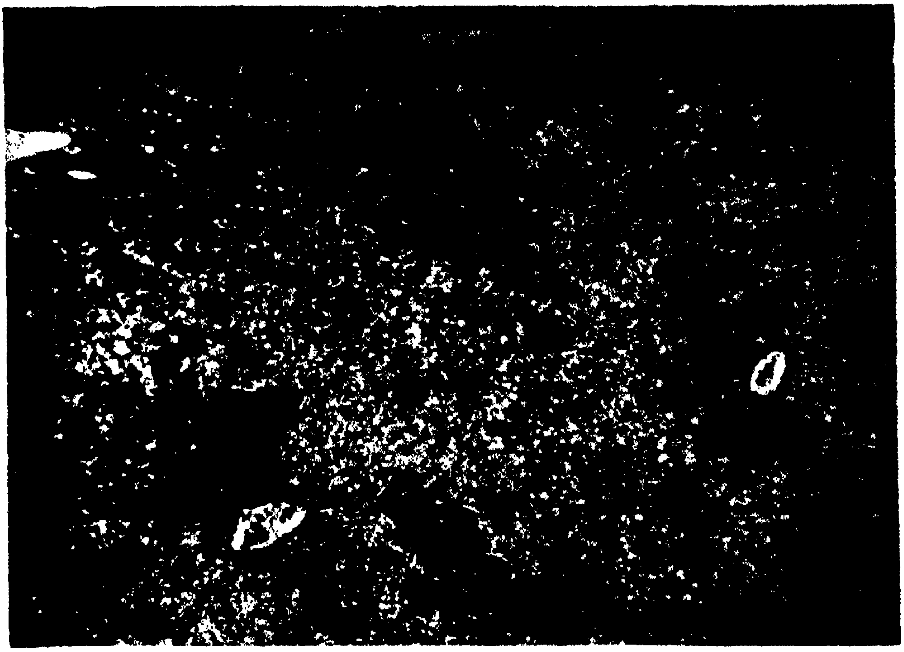
Diet	Animals dying with acute massive necrosis		Animals showing postnecrotic scarring	Total incidence of necrosis	
	Number	Average days on diet		Number	Percentage
H_2	5	85	3	8	80
H_3	7	77	3	10	100
A_1	3	60	1	4	40
A_3	1	70	—	1	10

All animals on diet H_2 died before the end of the experiment, five showing early acute massive necrosis; three, postnecrotic scarring; the remaining two, no demonstrable hepatic lesions. The rats on the H_3 diet also died during the experimental period. All presented evidence of liver necrosis, seven showing acute massive necrosis and three postnecrotic scarring.

Diet A_3 differed from A_1 only in the substitution of 0.01% cod liver oil concentrate for 2% cod liver oil. One animal in the former group showed necrosis, whereas four on A_1 diet displayed this lesion. All animals receiving a diet similar to A_1 but containing 8% casein survived the experiment and, at necropsy, showed no hepatic pathology.

The results obtained in this work indicate that acute massive hepatic necrosis can be induced readily in rats by feeding diets low in protein, a significant characteristic of which is, presumably, a deficiency in the sulphur-containing amino acids. It now appears definite, however, that factors other than those just mentioned play a role in the production of liver necrosis. Previous reports by Schwarz (15) and Gyorgy (4) indicated that tocopherols in the diet decreased the incidence of this lesion. This evidence has been confirmed by Himsworth and Lindan (8) and by Gyorgy and Goldblatt (5) in articles published recently. Thus any condition that effects the destruction or inhibition of tocopherols should accentuate the incidence of necrosis in animals on diets low in protein. It may be recalled that both cod liver oil (11) and rancid fats (3, 12) have been shown to exert an antagonistic effect on the

PLATE I



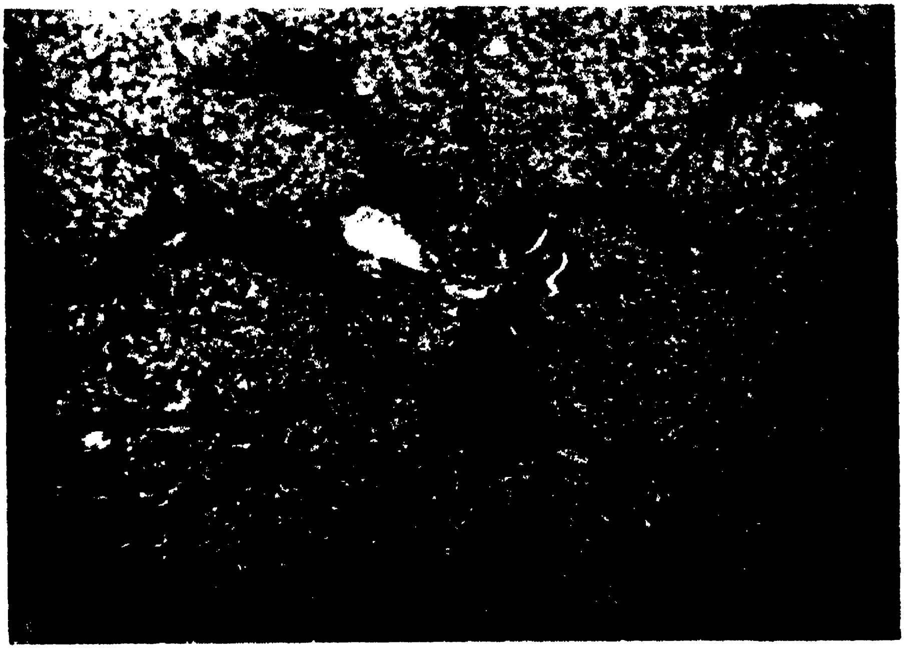


FIG. 1. *Acute massive hepatic necrosis.* ×70.

FIG. 2. *Postnecrotic scarring.* ×70.

action of dietary tocopherols. An examination of the data in Table II reveals that the incorporation of rancid fat (H_3) caused necrosis in all experimental animals whereas a similar diet with fresh lard (H_2) produced an incidence of 80%. The inclusion of cod liver oil (Diet A_1) led to a greater frequency of necrosis than did a similar diet (Diet A_3) containing a cod liver oil concentrate (40% vs. 10% respectively). These differences, although not statistically significant, at least suggest that any treatment that inhibits the action of tocopherols augments the capacity of the diet to produce necrosis.

Many workers have failed to induce acute massive hepatic necrosis, and indeed some have doubted the existence of such an entity, the cause of which could be attributed solely to dietary deficiencies. The foregoing work would suggest that, if the factors herein discussed are taken into consideration, a high incidence of necrosis can readily be produced.

Acknowledgments

This investigation was supported by a grant from the National Research Council of Canada.

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THE EFFECT OF SURGICAL OPERATIONS ON THE EXCRETION OF IODINE, CORTICOSTEROIDS, AND URIC ACID[1]

By W. F. Perry and J. P. Gemmell

Abstract

The effect of surgical operations on the urinary excretion of iodine, corticosteroids, uric acid, and ketosteroids was examined in 11 subjects. The majority showed a marked rise in the excretion of iodine which occurred within the first 24 hr. after operation; thereafter iodine excretion tended to decline, being back to normal levels by the third postoperative day. Over the same period of time the excretion of corticosteroids increased and was still elevated on the third postoperative day. The excretion of uric acid was elevated in some but not all of the subjects while no consistent trend in any direction was noted as regards the excretion of ketosteroids. The level of inorganic plasma iodine was elevated one to two hours postoperatively, while the level of protein bound plasma iodine was unchanged. No conclusion was reached concerning either the source of the extra iodine or the relationship between the increased excretion of iodine and the increased excretion of corticosteroids.

Introduction

As part of a study of thyroid and adrenal function it was decided to investigate the effect of trauma on the excretion of iodine and substances of adrenal cortical origin. This decision was prompted by the following considerations.

Some years ago McCullough (8) found an increase in the concentration of total iodine in the plasma following exercise and surgical operations. At about the same time Curtis and Phillips (1) demonstrated that thyroidectomy was associated with an increased excretion of iodine in the urine which commenced shortly after operation and persisted for some days; a similar phenomenon was encountered in relation to a small number of nonthyroid operations. Since iodine metabolism is intimately linked with the function of the thyroid gland, these findings suggested that trauma and stress may have some influence on the thyroid gland. Further evidence in support of such a concept has been supplied by White and Dougherty (15) who found that the catabolism of tissue protein following the stress of fasting was greatly diminished in the absence of the thyroid gland. It has also been frequently demonstrated that surgical operations and other damaging agents cause hypertrophy and increased activity of the adrenal cortex. One of the manifestations of this response is an increased urinary excretion of substances having biological and chemical properties similar to those of the 11 oxysteroids of the adrenal cortex (6, 12, 14, 15). The purpose of this paper is to present the results of concurrent estimations of the effects of trauma on the excretion of iodine and adrenal cortical derivatives.

As the level of protein bound plasma iodine is considered to reflect thyroid activity, the effect of trauma on this value was also investigated. In addition

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[1] *Manuscript received July 28, 1949.*

Contribution from the Department of Medicine and the Department of Physiology and Medical Research of the University of Manitoba, and from the Winnipeg General Hospital, Winnipeg, Man.

the opportunity was taken to observe the effect of trauma on the excretion of uric acid, since Forsham *et al.* (3) have shown the excretion of this substance is increased by the administration of certain adrenal cortical hormones and by stimulation of the cortex by its trophic hormones. It was thought that uric acid excretion might provide an easily measured index of adrenal cortical activity.

Subjects and Methods

The subjects of this investigation were selected so that only those free from acute illness and without disorders of thyroid function were studied. These subjects were in hospital for elective surgical procedures and with two exceptions showed no evidence of chronic ill health. One of these had chronic osteomyelitis while the other had mild diabetes. The subjects are listed in Table I. To avoid possible distortion of results by blood transfusions, the subjects were limited to those who were not expected to require transfusions and hence only moderate degrees of trauma were observed. However, one patient (No. 8) suddenly went into severe shock at operation and received blood and saline. No iodine preparations were used in these patients prior to, during, or following operation. Standard ward diets, modified by the use of uniodized salt and the exclusion of foods of high iodine content, were consumed during the experimental period.

All urine was collected in ice packed containers using no preservative and stored in the frozen state until analyzed. Precautions were taken to avoid contamination with iodine.

Total and protein bound plasma iodine values were determined on fasting venous blood by the method of Perry and Cosgrove (9). Inorganic plasma iodine was calculated by subtracting the protein bound from the total plasma iodine. This indirect determination of inorganic iodine was probably accurate to within ± 1.0 μgm. % of the reported value. The same procedure was used for urinary iodine with the following modifications. Urine was treated with activated charcoal and filtered. To 10 ml. of filtrate was added 300 mgm. of iodine free protein; the remainder of the procedure was the same as that used for plasma.

As indices of adrenal cortical activity, the urinary excretion of corticosteroids and ketosteroids was determined. Ketosteroids were determined colorimetrically by the usual reaction with *m*-dinitrobenzene in an alkaline medium to produce a red pigment. Corticosteroids were determined utilizing principles of the methods of Heard and Sobel (5) and of Talbot *et al.* (12). An aliquot of 100 ml. of urine was diluted with an equal volume of water and the pH adjusted to 1.0 with 12 N sulphuric acid and extracted three times with a cold 4 : 1 mixture of ether–chloroform. The extracts were pooled and washed with cold 0.1 N sodium hydroxide followed by cold water, dried with sodium sulphate, filtered, and evaporated at room temperature under reduced pressure. The residue was taken up in benzene and extracted several times with water. The pooled aqueous extracts were acidified to pH 2.0 with

TABLE I

Details of subjects and operations

No.	Sex	Age	Operation		
			Type	Duration, min.	Anesthetic
1	M	41	Plate to ulna and radius	30	Pentothal Curare
2	M	47	Arthrodesis of foot	60	Pentothal Cyclopropane
3	M	62	Removal of Smith-Petersen nail	60	Pentothal
4	M	35	Bone graft	90	Pentothal Curare
5	F	21	Removal medial meniscus	30	Pentothal
6	M	44	Removal medial meniscus	50	Curare Pentothal Ether Nitrous oxide
7	M	54	Osteotomy left arm	90	Pentothal Cyclopropane Nitrous oxide
8	M	47	Removal of sequestrum	80	Pentothal Cyclopropane
9	M	45	Removal of two screws from femur	60	Pentothal Ether
10	M	36	Removal of sequestra from hand Removal of plate from ulna	30	Pentothal
11	M	20	Excision of metatarsals and bunions	90	Curare Pentothal Cyclopropane
12	F	30	Dilatation cervix; suspension of uterus	90	Pentothal Curare
13	F	64	Excision nontoxic thyroid adenoma	40	Cyclopropane Nitrous oxide
14	M	16	Manipulation of hip	15	Pentothal

sulphuric acid and extracted four times with chloroform. The combined chloroform extracts were taken to dryness under reduced pressure. The residue was dissolved in 0.5 ml. of glacial acetic acid and its reducing properties measured using arsenomolybdic acid as a substrate and desoxycorticosterone as a reference standard. By this procedure the 24 hr. excretion of corticosteroids by normal adults of either sex has been found to be equivalent in reducing power to 0.40 to 0.85 mgm. of desoxycorticosterone. Duplicates agreed to within 0.05 mgm. per 24 hr. Uric acid was determined by the method of Benedict and Frank as described in Hawk, Oser, and Summerson (4).

Results

The 24 hr. urinary excretion of corticosteroids, ketosteroids, iodine, and uric acid of 11 subjects together with the mean excretions of these same subjects are shown in Fig. 1. In the majority of the 11 subjects there was an increased output of corticosteroids following operation. In the 10 subjects examined the excretion of ketosteroids was variable, in some instances being depressed after operation, in others elevated, so that no consistent change could be demonstrated during the time the observations were carried out. The excretion of uric acid in six of 10 subjects was elevated postoperatively; in the others no marked change was noted. Nine of the 11 subjects showed marked postoperative increases in the excretion of iodine in the urine, one a slight rise, and one no change. In one instance determinations of iodine were performed on the urine passed at intervals in the first 24 hr. following operation. These showed increased amounts of iodine in all specimens including the first passed at six hours.

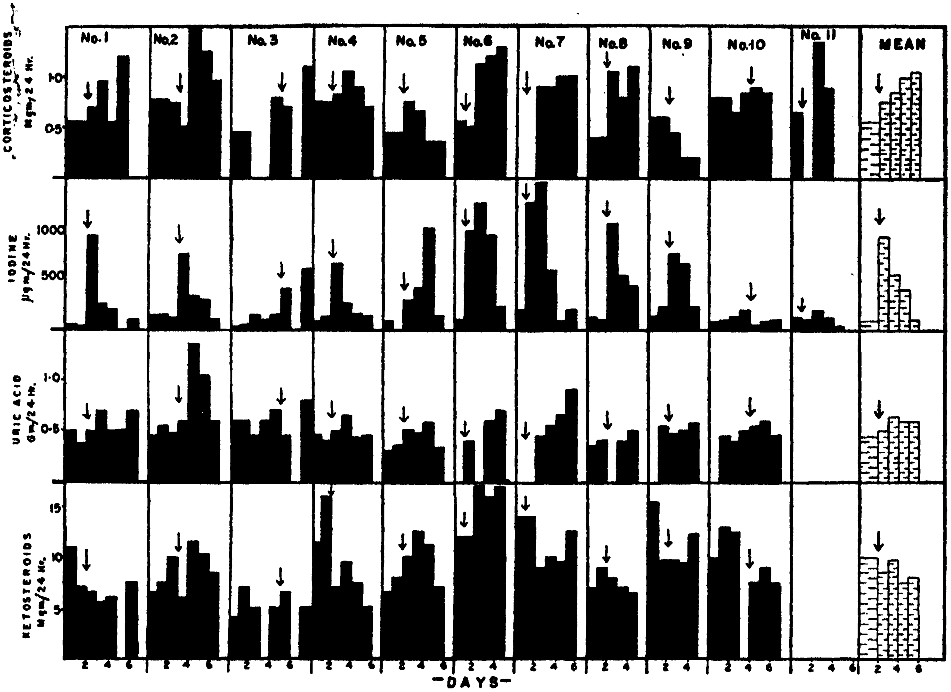

FIG. 1. *The effect of surgical operations on the excretion of corticosteroids, iodine, and uric acid. Day of operation indicated by a downward pointing arrow.*

Fig. 1 shows also that whereas by the third day following operation the excretion of corticosteroids was still elevated, the excretion of iodine was approaching the preoperative normal levels. That the effect of operative stress on iodine excretion is not a general one for halogen elements is indicated by the finding in three of the patients (Nos. 1, 2, 3) that the urinary chlorides were either unchanged or somewhat depressed. This is in agreement with Wilkinson *et al.* (16) who have reported a decline in the excretion of chlorides following operation.

The inorganic and protein bound plasma iodine values of nine subjects are listed in Table II. There was no significant change in the protein bound iodine in plasma taken at various times up to 96 hr. after operation. In eight of the nine cases in which it was studied there was an increase in plasma inorganic iodine within two hours of operation and by the second or third postoperative day relatively normal values were obtained.

TABLE II

EFFECT OF TRAUMA ON PLASMA IODINE (μGM./100 ML.)

No.	Inorganic				No.	Protein bound		
	Pre-operative	Hours after operation				Pre-operative	Hours after operation	
		1 to 2	8 to 12	24 to 96			1 to 2	24 to 96
1	0.0	2.4	1.4	0.5	1	4.8	—	6.5
2	0.0	0.0	1.0	0.0	3	4.0	4.0	—
3	1.0	5.2	—	0.6	5	4.4	4.4	3.2
5	—	2.2	—	0.5	6	5.8	5.8	—
7	0.5	3.0	—	0.9	7	4.9	5.4	4.3
9	0.0	3.2	—	0.9	9	3.6	—	5.5
10	0.5	1.5	—	—	10	5.5	—	6.4
11	2.0	2.8	1.5	1.8	11	4.5	—	4.2
12	0.0	14.1	—	4.5	12	4.0	4.3	4.4
					13	5.9	6.0	5.5
					14	7.4	7.0	5.7
Mean	0.5	3.9	1.3	1.2	Mean	5.0	5.2	5.0

Discussion

The above results show that surgical trauma results in increased activity of the adrenal cortex as indicated by increased excretion of corticosteroids. This is in agreement with the observations of other workers (6, 12, 10, 14, 15). No characteristic alteration in the excretion of 17 ketosteroids was observed, which is in disagreement with the results of Forbes *et al.* (2) and of Stevenson *et al.* (11) who reported a post-traumatic increase in the excretion of ketosteroids followed by a decline. Possibly the lack of agreement is due to differences in severity and type of trauma. It will be recalled that the majority of subjects in the present series were considered to have undergone relatively mild stress.

The hope that the estimation of uric acid excretion would provide an index of adrenal cortical activity was not fulfilled. Although the excretion of this substance was elevated in a number of patients following operation, in others there was no obvious change despite an increase in corticosteroid excretion.

From the above results it also appears that the trauma of surgical operations, including the effects of anesthesia, is associated with an increased excretion of iodine in the urine together with an elevation in the level of inorganic iodine in the plasma. The source of the extra iodine is not obvious. Possible sources are the thyroid gland, the inorganic iodine of the tissues, accelerated breakdown of thyroid hormone in the peripheral tissues and decreased pickup or utilization of inorganic iodine by the thyroid gland. The evidence available does not justify a detailed consideration of these possibilities, although the absence of any changes in the level of protein bound plasma iodine after trauma would suggest that thyroid function is not altered. However, it is possible that there is an increased utilization of thyroid hormone in the tissues with subsequent increased secretion by the thyroid, the net effect being a more or less constant level of hormonal iodine in the circulating plasma.

In considering the relationship which the increased iodine excretion bears to the increased excretion of corticosteroids it will be recalled that corticosteroid excretion is increased by a variety of stresses—including surgical operations. Exercise may also cause hypertrophy of the adrenal cortex (7) and increased excretion of cortical hormones (14). Preliminary observations indicate that an elevation of plasma and urinary inorganic iodine follows exercise in humans. Such similarities in response might suggest that they are closely related. However, the observations reported here concerning the increased iodine and corticosteroid excretion do not warrant any conclusion as to their causal relationship other than that they both result from trauma. In fact, the different patterns of excretion of iodine and of corticosteroids suggest that the increases are independent of one another.

Acknowledgments

The work reported in this paper was supported by a grant-in-aid to W.F.P. from the Division of Medical Research of the National Research Council, Canada, and was performed while J.P.G. held a Fellowship of the National Research Council. The authors are indebted to Dr. J. Doupe for advice and criticism and to Mrs. M. Court and Miss C. Anderson for technical assistance. Desoxycorticosterone and androsterone, used as standards in the analytical methods, were kindly donated by the Ciba Co. Ltd., and by Charles E. Frosst and Co., both of Montreal.

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CHOLINESTERASES OF CEREBROSPINAL FLUID[1]

DATA FOR NORMAL FLUIDS AND FLUIDS FROM PATIENTS WITH SYPHILIS, MENINGITIS, OR POLIOMYELITIS[1]

BY K. G. COLLING AND R. J. ROSSITER

Abstract

In confirmation of the work of others it has been shown that the principal enzyme in human cerebrospinal fluid that can hydrolyze acetylcholine is true cholinesterase. There is in addition a lesser quantity of pseudocholinesterase. The true cholinesterase, but not the pseudocholinesterase, was found to be significantly increased in the spinal fluids of patients with syphilis, while the pseudocholinesterase, and not the true cholinesterase, was increased in the fluids of patients with either meningitis or poliomyelitis. The true cholinesterase activity was correlated neither with the protein concentration nor with the cell count, while the pseudocholinesterase was correlated with the protein concentration and less significantly with the cell count. When correction was made for the correlation between cell count and protein concentration, the correlation between pseudocholinesterase activity and protein concentration remained statistically significant, but that between pseudocholinesterase and cell count became of dubious significance. In pathological conditions it appears unlikely that either true cholinesterase or pseudocholinesterase is derived from the white cells in the fluid. It is possible that the increased pseudocholinesterase comes from the blood plasma as a result of an increase in the permeability of the "plasma–spinal fluid barrier" and that the increased true cholinesterase comes from the substance of the brain or spinal cord. For the pathological conditions studied, the determination of true and pseudocholinesterase activity of the spinal fluid would be of little value as a diagnostic aid.

Introduction

Previous publications from this laboratory have been concerned with the enzymes of white blood cells (7, 21). In these studies white cells of animal origin only were used. Since pathological cerebrospinal fluid seemed a possible source of human white cells, we have engaged upon a systematic study of the enzymes, including the cholinesterases, of normal cerebrospinal fluids and fluids from patients with various pathological conditions. During the course of the investigation we became aware of a similar study on cholinesterases by Tower and McEachern (26, 27). In some respects our experiments with the cholinesterases of the cerebrospinal fluid are a confirmation of the work of these authors, although we employed a different technique. In other respects our experiments are a continuation of their interesting work.

The evidence in the literature concerning the presence of a cholinesterase in the cerebrospinal fluid is conflicting. Vahlquist (28) and Stedman and Stedman (25) were unable to demonstrate the hydrolysis of acetylcholine by cerebrospinal fluid, but Plattner and Hintner (19), Altenburger (2), and Bender (4), using biological methods of acetylcholine assay, and Birkhauser

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[1] *Manuscript received July 11, 1949.*

Contribution from the Department of Biochemistry and Division of Dermatology, Faculty of Medicine, University of Western Ontario, London, Ont.

The work was supported by grants from the Ontario Department of Education and The National Research Council of Canada.

(5) and Glasson and Mutrux (9), using the manometric method of Ammon (3), all claimed that the cerebrospinal fluid is able to hydrolyze acetylcholine. The recent publications of Tower and McEachern (27) and Reiss and Hemphill (20) have established beyond any doubt that this is so.

It is now generally recognized that there are at least two enzymes in the body capable of hydrolyzing acetylcholine: true cholinesterase, which can hydrolyze acetyl-β-methylcholine and not benzoylcholine, and pseudocholinesterase, which can hydrolyze benzoylcholine and not acetyl-β-methylcholine (1, 13, 14, 18). These enzymes are sometimes called the "specific" and the "non-specific" cholinesterase respectively (10). On indirect evidence Glasson and Mutrux (9) concluded that the enzyme of the cerebrospinal fluid that is able to hydrolyze acetylcholine is chiefly true cholinesterase, a result confirmed by Tower and McEachern (27) and Reiss and Hemphill (20), using the substrate specificity test of Mendel (13). A small quantity of pseudocholinesterase is also present.

Methods

Since we were measuring the activity of a number of enzymes and the amount of spinal fluid available was limited, the manometric method was not suited to our purpose. We used, therefore, a microtitration procedure that required only 0.4 ml. fluid (0.2 ml. for the test and 0.2 ml. for the blank). The final concentration of the substrate was: acetylcholine 0.005 *M*, acetyl-β-methylcholine 0.025 *M*, and benzoylcholine 0.005 *M*. In confirmation of the report of Mendel *et al.* (13) this concentration of acetyl-β-methylcholine and benzoylcholine was shown to be optimal. Since the optimal concentration of acetylcholine for pseudocholinesterase differs from that for true cholinesterase (13, 18), the optimal concentration of this substrate for any given cerebrospinal fluid will depend upon the relative concentration of these two enzymes that is present. At first observations were made at three substrate concentrations, 0.05 *M*, 0.005 *M*, and 0.0005 *M*. These figures yielded little additional information and the figure of 0.005 *M* was arbitrarily selected for the remainder of the work.

To a pyrex glass tube was added:

2 ml. diethylbarbiturate buffer pH 7.2 (16) diluted with an equal volume of water.

0.2 ml. substrate (either 0.06 *M* acetylcholine, 0.3 *M* acetyl-β-methylcholine, or 0.06 *M* benzoylcholine*).

0.2 ml. cerebrospinal fluid.

The tube was incubated in a 37° C. water bath for 18 hr. At the end of this time the tube was removed from the bath and two drops of mixed indicator (equal parts of 33 mgm. bromcresol green per 100 ml. ethyl alcohol and 66 mgm. methyl red per 100 ml. ethyl alcohol) added. Hydrochloric acid 0.003 *N* was then added carefully from a 5 ml. microburette until the sharp green-to-red end point was reached. A blank, in which substrate and buffer were incubated for 18 hr. and cerebrospinal fluid added just before the titration, was also set up and titrated in the same way. The difference between the titration figure for the blank and that for the test is a measure of the enzyme activity.

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* *Kindly provided by Hoffmann—La Roche Limited.*

If the difference between the titration figure of the blank and that of the test is *a* ml., the enzyme activity (expressed as 0.001 *N* acid per 100 ml. cerebrospinal fluid per hr.)

$$= a \times \frac{0.003}{0.001} \times \frac{100}{0.2} \times \frac{1}{18}$$

$$= a \times 83.3$$

This figure can be converted to the perhaps more familiar ml. carbon dioxide per ml. per hr. by multiplying by the factor 0.224. A drop of toluol was added to the total volume of the fluid immediately after it was collected. Both test and blank were always run in duplicate. Such an experimental procedure is not ideal because the activity of the enzyme was not strictly linear with time for 18 hr. For this reason our figures are not comparable with those reported by other workers (20, 27). Since this long incubation period was necessary if reasonable titration figures were to be obtained, some measure of compromise seemed desirable. Fig. 1 shows, however, that under the conditions of study the titration figure was proportional to the concentration of the enzyme.

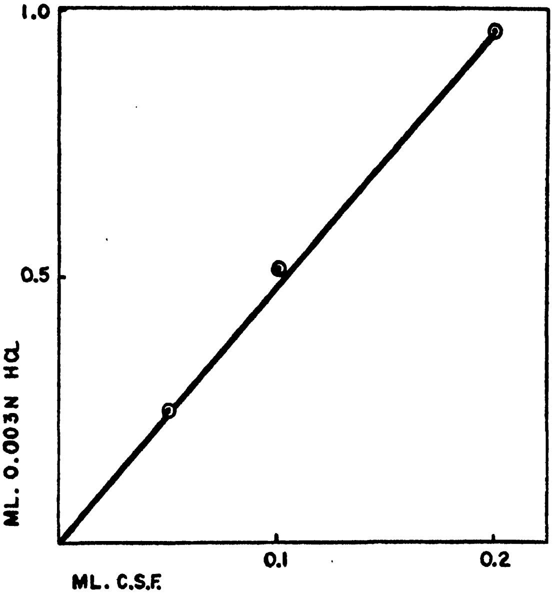

FIG. 1. *Relation between enzyme activity and quantity of cerebrospinal fluid.*

Results

Cholinesterases of Blood

Table I shows that both plasma and lysed red cells from human blood hydrolyzed acetylcholine. Whereas plasma hydrolyzed benzoylcholine and little acetyl-β-methylcholine, the lysed red cells hydrolyzed acetyl-β-methylcholine and practically no benzoylcholine. This is in direct confirmation of the work of Mendel (13, 14) and demonstrates that it is possible to distinguish between the true cholinesterase of the red cells and the pseudocholinesterase of the plasma by the microtitration method.

TABLE I

HYDROLYSIS OF CHOLINE ESTERS BY HUMAN PLASMA AND LYSED RED BLOOD CELLS (ML. 0.001 *N* ACID PER ML. PER HR.)

Substrate	Number of observations	Plasma		Lysed red blood cells	
		Mean	S.E. mean	Mean	S.E. mean
Acetylcholine 0.005 *M*	5	82.3	±11.6	104.1	±9.7
Acetyl-β-methylcholine 0.025 *M*	5	8.7	± 1.3	84.5	±4.3
Benzoylcholine 0.005 *M*	5	81.9	± 7.5	0.75	±0.75

Cholinesterases of Normal Cerebrospinal Fluids

Tables II, III, and IV show the hydrolysis of acetylcholine, acetyl-β-methylcholine, and benzoylcholine by normal cerebrospinal fluids. These fluids were taken from apparently healthy adults who were under 55 years of age and in whom there was no history of systemic disease, such as syphilis, tuberculosis, or diabetes. Each specimen had a normal protein concentration and cell count, and negative Wassermann and colloidal gold reactions. Many of the fluids were withdrawn from the subjects before the injection of a spinal anaesthetic for a "cold" surgical operation.

The cholinesterase activity of the cerebrospinal fluid was, on the average, 0.70% of that of blood serum. Reiss and Hemphill (20) found that the cholinesterase activity of cerebrospinal fluid varied from 0.67% to 1.11% of that of serum, while Tower and McEachern (27) reported a mean value of 0.45%. The observation that cerebrospinal fluid hydrolyzed acetyl-β-methylcholine more rapidly than benzoylcholine agrees with the findings of both groups of workers. The rate of hydrolysis of acetyl-β-methylcholine was 42% and that of benzoylcholine 23% of the rate of hydrolysis of acetylcholine. This can be compared with the respective figures of 52% and 19.5% reported by Reiss and Hemphill (20) and 33% and 17% reported by Tower and McEachern (27), both these groups using the manometric method.

TABLE II

HYDROLYSIS OF 0.005 *M* ACETYLCHOLINE BY CEREBROSPINAL FLUID (ML. 0.001 *N* ACID PER 100 ML. PER HR.)

Diagnosis	No. patients	Mean	S.E. of mean	*P*
Normal	14	57.4	± 3.7	—
Syphilis (negative C.S.F. Wassermann)	17	71.8	± 4.7	*<0.05*
Syphilis (positive C.S.F. Wassermann)	16	62.9	± 5.7	>0.4
Meningitis	10	93.6	±12.9	*<0.01*
Poliomyelitis	8	59.6	± 8.1	>0.7

TABLE III

HYDROLYSIS OF 0.025 *M* ACETYL-β-METHYLCHOLINE BY CEREBROSPINAL FLUID (ML. 0.001 *N* ACID PER 100 ML. PER HR.)

Diagnosis	No. patients	Mean	S.E. of mean	*P*
Normal	18	24.3	±1.6	—
Syphilis (negative C.S.F. Wassermann)	24	36.4	±3.4	*<0.01*
Syphilis (positive C.S.F. Wassermann)	30	34.2	±3.2	*<0.02*
Meningitis	11	22.2	±2.6	>0.4
Poliomyelitis	25	32.5	±4.3	>0.1

TABLE IV

HYDROLYSIS OF 0.005 *M* BENZOYLCHOLINE BY CEREBROSPINAL FLUID (ML. 0.001 *N* ACID PER 100 ML. PER HR.)

Diagnosis	No. patients	Mean	S.E. of mean	*P*
Normal	19	13.4	±1.2	—
Syphilis (negative C.S.F. Wassermann)	23	15.8	±1.8	>0.2
Syphilis (positive C.S.F. Wassermann)	30	15.5	±2.0	>0.4
Meningitis	11	81.4	±1.6	*<0.01*
Poliomyelitis	23	25.3	±3.5	*<0.01*

Syphilis

Determinations were done on the cerebrospinal fluids from a series of patients with syphilis (positive blood Wassermann reaction). The fluids were divided into two groups according as to whether the spinal fluid Wassermann reaction was positive or negative. Table II shows that the mean rate of hydrolysis of acetylcholine by the fluids with a positive Wassermann reaction was not significantly greater than that of the normal fluids ($P > 0.4$) and that the rate of hydrolysis of acetylcholine by fluids with a negative Wassermann reaction was only slightly greater ($P < 0.05$). The hydrolysis of acetyl-β-methylcholine was significantly greater for both groups (Table III), while there was no significant difference in the hydrolysis of benzoylcholine (Table IV). There was thus a significant increase in the mean true cholinesterase activity in the cerebrospinal fluid of patients with syphilis and no change in the pseudocholinesterase activity. This increase was independent of the cerebrospinal fluid Wassermann reaction.

Tables V, VI, and VII give the number of patients from whom were taken the fluids that showed an increase in hydrolysis of acetylcholine, acetyl-β-methylcholine, or benzoylcholine for each of the conditions studied. An increase in the enzyme activity was defined as an activity that exceeded the mean of the normal fluids by twice the standard deviation. For the enzyme activity of a particular fluid to be reported as increased the figures would therefore have to exceed 85.2 ml. 0.001 *N* acid per 100 ml. per hr. for acetylcholine, 37.8 for acetyl-β-methylcholine, and 23.8 for benzoylcholine. Three of the 17 patients with syphilis and a negative spinal fluid Wassermann reaction, and two of the 16 patients with syphilis and a positive spinal fluid Wassermann

TABLE V

ACETYLCHOLINE HYDROLYSIS BY CEREBROSPINAL FLUID AND CLINICAL DIAGNOSIS

Diagnosis	No. patients	No. with normal acetylcholine hydrolysis	No. with increased acetylcholine hydrolysis	Percentage with increased acetylcholine hydrolysis, %	χ^2	*P*
Normal	14	14	0	0	—	—
Syphilis (negative C.S.F. Wassermann)	17	14	3	18	2.07	>0.1
Syphilis (positive C.S.F. Wassermann)	16	14	2	13	1.87	>0.1
Meningitis	10	4	6	60	8.03	*<0.01*
Poliomyelitis	8	7	1	13	1.83	>0.1

TABLE VI

TRUE CHOLINESTERASE IN CEREBROSPINAL FLUID AND CLINICAL DIAGNOSIS

Diagnosis	No. patients	No. with normal true cholinesterase	No. with increased true cholinesterase	Percentage with increased true cholinesterase, %	χ^2	*P*
Normal	18	18	0	0	—	—
Syphilis (negative C.S.F. Wassermann)	24	15	9	38	8.59	*<0.01*
Syphilis (positive C.S.F. Wassermann)	30	22	8	27	5.75	*<0.02*
Meningitis	11	11	0	0	0.00	>0.99
Poliomyelitis	25	19	6	24	5.02	<0.05

TABLE VII

PSEUDOCHOLINESTERASE IN CEREBROSPINAL FLUID AND CLINICAL DIAGNOSIS

Diagnosis	No. patients	No. with normal pseudocholinesterase	No. with increased pseudocholinesterase	Percentage with increased pseudocholinesterase, %	χ^2	*P*
Normal	19	19	0	0	—	—
Syphilis (negative C.S.F. Wassermann)	23	18	5	22	4.68	<0.05
Syphilis (positive C.S.F. Wassermann)	30	25	5	17	3.52	>0.05
Meningitis	11	2	9	82	22.2	*<0.01*
Poliomyelitis	23	14	9	39	9.46	*<0.01*

reaction had an increased acetylcholine hydrolysis (Table V). A χ^2 test showed that this was not significantly different from the control group. Table VI shows that, of the patients with syphilis, only nine of the 24 with a negative spinal fluid Wassermann reaction and only eight of the 30 with a positive spinal fluid Wassermann reaction had a true cholinesterase activity that could be reported as increased i.e. an activity that exceeded the mean of that of the normal fluids by more than twice the standard deviation. Although a χ^2 test indicated that these figures are significantly different from those of the normal fluids, the small percentage of fluids with an increased true cholinesterase activity would indicate that the test is of little value from the diagnostic point of view. Also only five of the 23 patients with a negative spinal fluid Wassermann reaction and five of the 30 with a positive spinal fluid Wassermann reaction had an increased pseudocholinesterase activity.

Meningitis

Table II shows that the hydrolysis of acetylcholine by the spinal fluids of patients with meningitis was increased ($P < 0.01$). The subjects studied included two with meningococcal meningitis, five with pneumococcal meningitis, and three with tuberculous meningitis. Table III shows that the increase in acetylcholine hydrolysis in spinal fluids of patients with meningitis was not accompanied by an increase in the hydrolysis of acetyl-β-methylcholine ($P > 0.4$), but Table IV shows that there was a great increase in the hydrolysis of benzoylcholine ($P < 0.01$). Thus in the spinal fluids of patients with meningitis, there was an increase in pseudocholinesterase activity with no change in true cholinesterase activity.

Of the 10 fluids from patients with meningitis in which the hydrolysis of acetylcholine was studied, six had an increased acetylcholine hydrolysis (Table V) while in none of the 11 fluids in which the hydrolysis of acetyl-β-methylcholine was studied was there an increase in the true cholinesterase activity (Table VI). On the other hand, nine of the 11 fluids in which the hydrolysis of benzoylcholine was studied had a pseudocholinesterase activity greater than the mean of the normal group by more than twice the standard deviation (Table VII). The two meningitic fluids that did not show increased hydrolysis of benzoylcholine were from patients who had received numerous intrathecal injections of antibiotic drugs before the specimens were taken. In many of the meningitic patients repeated spinal fluid examinations were made for pseudocholinesterase activity. These also revealed an increased benzoylcholine hydrolysis. With clinical improvement, accompanied by a decrease in spinal fluid cell count and protein concentration, the hydrolysis of benzoylcholine decreased. The findings of the first test only for each patient are reported in Tables II to VII. There appeared to be no association between the type of cell predominating in the fluid and the pseudocholinesterase activity.

Poliomyelitis

Table II shows that there was no significant increase in the hydrolysis of acetylcholine in fluids from patients with established poliomyelitis ($P > 0.7$). Although Table III shows that there was also no significant increase in the hydrolysis of acetyl-β-methylcholine ($P > 0.1$), Table IV shows that there was a statistically significant increase in the hydrolysis of benzoylcholine ($P < 0.01$).

Of the 25 fluids from patients with poliomyelitis in which the hydrolysis of acetyl-β-methylcholine was studied, six had a significant increase in true cholinesterase (Table VI) and, of the 23 fluids in which the hydrolysis of benzoylcholine was studied, nine had an increase in pseudocholinesterase activity (Table VII). In both instances the figures were significant as judged by the χ^2 test, but the percentage of fluids whose enzyme activity exceeded that of the normal fluids by more than twice the standard deviation was too low for the test to be considered of any diagnostic value.

Concentration of Protein

Table VIII gives the coefficient of correlation between the concentration of protein and the hydrolysis of each of the choline esters studied. In compiling these statistics, the figures for the choline ester hydrolysis of all fluids were pooled, regardless of the clinical diagnosis. There was a slight, but statistically significant ($r = 0.37 \pm 0.12$), correlation between the hydrolysis of acetylcholine and the protein concentration. This must have been entirely due to the pseudocholinesterase activity, for there was no significant correlation between the hydrolysis of acetyl-β-methylcholine ($r = -0.17 \pm 0.10$) and protein concentration, and a highly significant correlation between the hydrolysis of benzoylcholine ($r = 0.66 \pm 0.06$) and the protein concentration. This relation is shown in Fig 2.

TABLE VIII

CORRELATION BETWEEN THE HYDROLYSIS OF CHOLINE ESTERS BY CEREBROSPINAL FLUID AND THE CONCENTRATION OF PROTEIN

Substrate	Number of observations	Coefficient of correlation (r)	S. E. of r
Acetylcholine 0.005 *M*	53	0.37	±0.12
Acetyl-β-methylcholine 0.025 *M*	95	−0.17	±0.10
Benzoylcholine 0.005 *M*	94	0.66	±0.06

Cell Count

Table IX shows that a similar, though less significant, correlation existed between the hydrolysis of the choline esters and cell count. The correlation was just significant ($r = 0.31 \pm 0.11$) for acetylcholine, insignificant ($r = -0.05 \pm 0.09$) for acetyl-β-methylcholine, and of slightly greater significance for benzoylcholine ($r = 0.49 \pm 0.08$). From these figures one might be tempted to speculate that the correlation between the hydrolysis of benzoylcholine and the cell count might be due to pseudocholinesterase activity in the white cells. However, it was observed that most of the fluids with both a high benzoylcholine activity and a high cell count also had a high protein concentration and were usually from patients with meningitis. On the other hand, the fluids with a high cell count and normal benzoylcholine hydrolysis usually only had a slightly increased protein concentration and were frequently from patients with other conditions, e.g. poliomyelitis. The fluids were,

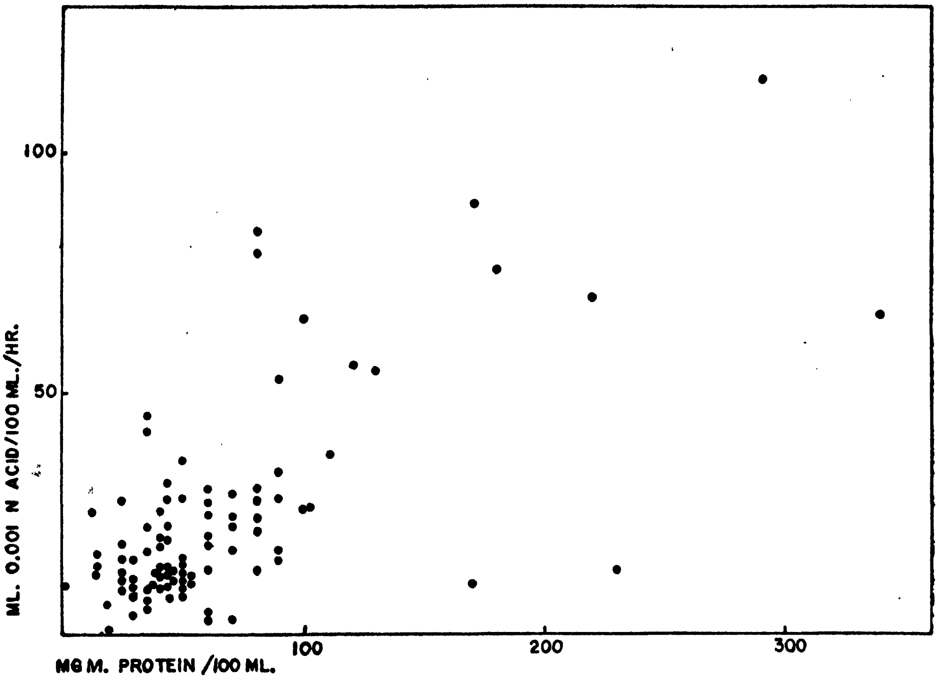

FIG. 2. *Relation between concentration of protein and pseudocholinesterase activity of human cerebrospinal fluids.*

therefore, divided into two groups: a small group of 19 fluids from patients with meningitis and a large group of 75 fluids from patients with other conditions. Table IX shows that the hydrolysis of benzoylcholine was just significantly correlated with cell count for the meningitic fluids ($r = 0.46 \pm 0.18$), whereas there was no such significant correlation for the larger group of 75 fluids from patients with other conditons ($r = 0.08 \pm 0.11$).

TABLE IX

CORRELATION BETWEEN THE HYDROLYSIS OF CHOLINE ESTERS BY CEREBROSPINAL FLUID AND THE CELL COUNT

Substrate	Number of observations	Coefficient of correlation (r)	S. E. of r
Acetylcholine 0.005 *M*	73	0.31	±0.11
Acetyl-β-methylcholine 0.025 *M*	95	−0.05	±0.09
Benzoylcholine 0.005 *M*	94	0.49	±0.08
Benzoylcholine (meningitis only)	19	0.46	±0.18
Benzoylcholine (nonmeningitic)	75	0.08	±0.11

It thus appeared that the correlation between benzoylcholine hydrolysis and cell count reported in Table IX ($r = 0.49 \pm 0.08$) was the result of a significant correlation between protein concentration and cell count, since protein concentration was shown previously to be correlated with benzoylcholine hydrolysis (Table VIII). Table X shows these three correlation coefficients together with the coefficient of partial correlation between pseudocholinesterase activity and protein concentration with cell count excluded and that between pseudocholinesterase activity and cell count with protein concentration excluded. It can be seen that with cell count excluded the pseudocholinesterase activity was still significantly correlated with protein concentration ($r_{12.3} = 0.54 \pm 0.07$), but that with protein concentration excluded the correlation coefficient between pseudocholinesterase activity and cell count ($r_{13.2}$) was only 0.21 ± 0.10. This figure is barely twice its standard error and is of extremely dubious significance.

TABLE X

CORRELATION COEFFICIENTS AND PARTIAL CORRELATION COEFFICIENTS BETWEEN THE PSEUDOCHOLINESTERASE ACTIVITY, PROTEIN CONCENTRATION, AND CELL COUNT OF 94 CEREBROSPINAL FLUIDS

	Correlation between	Coefficient of correlation	S.E. of r
r_{12}	Pseudocholinesterase activity and protein concentration	0.66	±0.06
r_{13}	Pseudocholinesterase activity and cell count	0.49	±0.08
r_{23}	Protein concentration and cell count	0.54	±0.07
$r_{12.3} = \dfrac{r_{12} - (r_{13} \times r_{23})}{\sqrt{1 - r_{13}^2} \times \sqrt{1 - r_{23}^2}}$	Pseudocholinesterase activity and protein concentration (cell count excluded)	0.54	±0.07
$r_{13.2} = \dfrac{r_{13} - (r_{12} \times r_{23})}{\sqrt{1 - r_{12}^2} \times \sqrt{1 - r_{23}^2}}$	Pseudocholinesterase activity and cell count (protein concentration excluded)	0.21	±0.10

From these data it can be concluded that cell count per se does not influence the hydrolysis of either acetyl-β-methylcholine or benzoylcholine by cerebrospinal fluids. A similar conclusion was reached by Tower and McEachern (27).

Discussion

In confirmation of the work of others (20, 27) cerebrospinal fluid would thus appear to contain a true cholinesterase, which is able to hydrolyze acetyl-β-methylcholine, and a pseudocholinesterase, which is able to hydrolyze benzoylcholine. The true cholinesterase predominates in normal fluids. There was the possibility that the enzyme in the cerebrospinal fluid that hydrolyzed benzoylcholine was not pseudocholinesterase, but the benzoylcholinesterase described by Sawyer (22) and Gunter (11). Gunter (11) reported that this enzyme was not inhibited by $2 \times 10^{-6}M$ eserine, a concentration of eserine that completely inhibited pseudocholinesterase. The cerebrospinal fluid enzyme was completely inhibited by an eserine concentration of $10^{-5}M$ while a concentration of $10^{-6}M$ produced almost complete inhibition. This would favor the view that the cerebrospinal fluid enzyme that hydrolyzes benzoylcholine is a pseudocholinesterase rather than a specific benzoylcholinesterase.

Kaplan, Cohn, Levinson, and Stern (12) suggested that enzymes in normal cerebrospinal fluid may be derived from either the tissue of the central nervous system, i.e. brain or spinal cord, or from the blood plasma. They also suggested that the enzyme activity in pathological conditions could be increased by one or more of the following three methods:

(*a*) An increase in enzyme-containing cells in the cerebrospinal fluid.

(*b*) An increase in the permeability of the membrane between the blood plasma and the cerebrospinal fluid, with the passage of plasma enzymes into the cerebrospinal fluid.

(*c*) A destruction of the tissue of the brain or spinal cord with the appearance of central nervous tissue enzymes in the cerebrospinal fluid.

The observation that the cell count per se does not influence the hydrolysis of either acetyl-β-methylcholine or benzoylcholine would indicate that the white cells are devoid of either true or pseudocholinesterase. Ginsberg, Kohn, and Necheles (8) found no cholinesterase activity in polymorphonuclear leucocytes from the pus of an empyema, while Rossiter and Wong (21) showed that the rabbit polymorphonuclear leucocyte is unable to hydrolyze acetylcholine, acetyl-β-methylcholine, or benzoylcholine. Also Brauer and Hardenbergh (6) reported that there is little cholinesterase in the lymphocyte and Youngstrom (29) stated that the enzyme is absent from the macrophages of a brain tumor. The absence of cholinesterase in white cells is unusual, for most tissues contain either true or pseudocholinesterase, or both (23). It would thus appear that Method (*a*) could not account for the increase in true cholinesterase observed in the spinal fluids of patients with syphilis or the increase in pseudocholinesterase observed in the fluids of patients with meningitis or poliomyelitis.

It is possible that the pseudocholinesterase of the cerebrospinal fluid comes from the blood plasma (Method (*b*)), for human plasma is rich in pseudocholinesterase and almost devoid of true cholinesterase (1, 13, 14, 18). The increase in pseudocholinesterase in the cerebrospinal fluid of patients with damaged meninges, i.e. meningitis, would support this view. It is also supported by the observation that the pseudocholinesterase activity of the cerebrospinal fluid is correlated with the protein concentration. Both could be the result of an increase in the permeability of the membrane between the blood plasma and the cerebrospinal fluid that may occur in meningitis (24).

Tower and McEachern (26) have suggested that a similar mechanism may explain the increase in pseudocholinesterase observed in the spinal fluids from patients with craniocerebral trauma.

Although Method (*b*) could account for the increase in pseudocholinesterase it could not account for the increase in true cholinesterase. On the other hand, Method (*c*) could account for the increase in true cholinesterase but not for the increase in pseudocholinesterase, since the brain and spinal cord are rich in true cholinesterase and almost devoid of pseudocholinesterase (13, 14, 15, 17, 18). That there is a significant increase in the true cholinesterase activity of fluids from patients suffering from syphilis, a disease that produces demonstrable damage to the central nervous system, is evidence in favor of this view.

There are few observations in the literature on the hydrolysis of acetylcholine by cerebrospinal fluids from patients with syphilis, meningitis, or poliomyelitis. Birkhauser (5) reported an increased hydrolysis of acetylcholine by spinal fluids from patients with either tuberculous or meningococcal meningitis. He was also unable to find any correlation between the cholinesterase activity and the white cell count of the fluids.

Cerebrospinal fluid cholinesterase determinations would appear to be of little value in the diagnosis of syphilis, meningitis, or poliomyelitis. Obviously a determination of the hydrolysis of acetylcholine would be of little use. Although the mean figure for the hydrolysis of acetyl-β-methylcholine by fluids from patients with syphilis was significantly greater than the mean figure for the normal fluids, the overlap was so great that the test would be of little diagnostic value. The same applies to the increase in the hydrolysis of benzoylcholine by fluids from patients with poliomyelitis. This test could be of use in the diagnosis of meningitis for, in nine of the 11 patients studied, the benzoylcholine hydrolysis was greater than the mean of the normal fluids, by more than twice the standard deviation. The test has, however, no obvious advantages over the established diagnostic procedures for this disease.

Acknowledgments

We wish to thank Dr. D. McEachern and Dr. D. B. Tower for reading this manuscript and for making a number of valuable suggestions. Thanks are also due to Dr. G. B. Sexton for his interest in the work and to Dr. W. Wilson for his willing cooperation. We also wish to thank Dr. H. T. Norry, Westminster Hospital, London, and those members of the Medical and Nursing Staff of Victoria Hospital who assisted in this work. Miss Ann Morrison and Miss Doris Grainger rendered skilful technical assistance.

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SOME OBSERVATIONS ON INTERFERENCE BETWEEN NEUROTROPIC VIRUSES[1]

BY A. J. RHODES AND MARION CHAPMAN

Abstract

Studies have been carried out on the interference phenomenon with certain neurotropic viruses in experimental animals. The primary object was to investigate claims that material containing human poliomyelitis virus interferes with the progress of the infection by the MM virus in hamsters, or the Lansing virus in mice. We have failed to find evidence that the inoculation of specimens known to contain human poliomyelitis virus interferes with the progress of MM or Lansing virus infections, and we do not believe that the interference phenomenon can be used in this way as a diagnostic test. In the course of this investigation, it was found that interference occurs when hamsters are inoculated cerebrally with lymphocytic choriomeningitis virus, and, four to seven days later, with the antigenically unrelated MM virus by the peritoneal route. A significant number of such treated animals are spared from developing paralysis due to the MM virus. Evidence was also obtained that under certain circumstances the inoculation of MM virus may lessen the severity of the illness due to lymphocytic choriomeningitis virus injected several days earlier.

Introduction

If two antigenically distinct viruses are inoculated into a single experimental animal at relatively short intervals, the phenomenon known as "interference" or "cell blockade" may be observed, the animal failing to develop signs of infection. This interference phenomenon has been extensively studied, and is known to occur with many viruses. We are here concerned with certain members of the group of neurotropic viruses. Thus, it has been claimed that the injection of lymphocytic choriomeningitis (LCM) virus in monkeys exerts a sparing effect on the development of paralysis from the subsequent inoculation of human poliomyelitis virus (1). A similar effect is said to be produced by the viruses known as Columbia-SK (6), and MM (3). It has also been shown that lymphocytic choriomeningitis virus exerts a sparing effect in hamsters later inoculated with MM virus (2), and it has been reported that interference can be demonstrated in guinea pigs between human poliomyelitis virus and MM virus (5). It is probable that the blocking action of the Columbia-SK and MM viruses is largely associated with the presence of infective virus particles, as preparations treated with chemicals do not act as interfering agents (3, 4).

The precise mechanism of the apparent interference with the propagation of human poliomyelitis virus is not known, although it is clear that there is no antigenic relationship between this virus and those used as interfering agents. Immunity mechanisms, as generally understood, do not therefore seem to be involved.

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[1] *Manuscript received August 3, 1949.*

Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont. Aided by a grant from the National Foundation for Infantile Paralysis.

Apart from the considerable theoretical interest of the interference phenomenon, a practical application might be found in the introduction of a presumptive diagnostic test for poliomyelitis virus, the presence of which can at present only be demonstrated by inoculation of monkeys. Dalldorf and Whitney (2) have in fact reported that the cerebral inoculation of stool extracts and cords infected with poliomyelitis virus in hamsters interferes with the propagation of MM virus. Experiments on the elaboration of a diagnostic test have also been reported by Lépine (7), who claimed that infected stools and cords interfered with the development of the Lansing strain of poliomyelitis in mice. This claim was, however, subsequently withdrawn (8).

The work reported in this paper was conducted in the first instance to investigate the above mentioned suggestions that the interference phenomenon might be of value in the laboratory diagnosis of poliomyelitis infection. Our studies do not suggest that use can be made of this reaction for the detection of poliomyelitis virus in human material. In the course of our work, however, we have found that interference occurs in hamsters inoculated with the lymphocytic choriomeningitis and MM viruses. This reaction appears worthy of study as a model of the interference phenomenon with neurotropic viruses in general.

Methods

Experiments have been conducted with golden hamsters and mice. The design of the experiments with hamsters was suggested by the report (2) that interference could be shown when an interval of about seven days separated a primary cerebral injection of an interfering agent, such as an extract of human stool or monkey cord, or lymphocytic choriomeningitis virus, and a later peritoneal inoculation of MM virus. The procedure in mice, suggested by the work of Lépine (7), involved the inoculation of nervous tissue infected with human poliomyelitis virus by the cerebral route, and 48 hr. later the challenge of these treated animals by the cerebral route with the Lansing strain of poliomyelitis virus.

Storage.—All pathological specimens and neurotropic viruses used in this study were preserved in the carbon dioxide ice chest (−60° C.).

Stools obtained from six patients diagnosed clinically as suffering from poliomyelitis were prepared as 10% watery extracts by grinding them in a mortar. After low speed centrifugation, these extracts were rendered bacteria-free in one of three ways: (1) By filtration through a Seitz filter. (2) By repeated shaking with one-third volume of ether, as described fully in another communication from this laboratory (9). (3) By differential centrifugation in an ultracentrifuge, the virus finally being deposited at a speed of 39,000 r.p.m. (170,000 times gravity). Five of the six stools were demonstrated to contain human poliomyelitis virus by inoculation of rhesus monkeys. Stool extracts were used only in experiments with hamsters, being injected cerebrally in 0.05 ml. amounts; they were mostly well tolerated, although the

TABLEAU VI

Traitement	Nombre de rats	Cholestérol, % (mg./100 mg.)	Cholestérol total (mg.)	Différences entre les groupes			
				Cholestérol, %		Cholestérol total	
				Différence	*t*	Différence	*t*
Ascorbate au froid	25	2.24 ±0.167*	0.395 ±0.031	−29.56	*3.67*	−27.65	*4.38*
Ascorbate à temp. normale	26	3.18 ±0.195	0.546 ±0.015				
Bicarbonate au froid	22	2.77 ±0.354	0.525 ±0.076	−10.64	0.68	− 1.50	0.10
Bicarbonate à temp. normale	24	3.10 ±0.329	0.533 ±0.033				
Témoins au froid	26	2.09 ±0.173	0.411 ±0.042	−33.43	*3.27*	−21.26	1.43
Témoins à temp. normale	25	3.14 ±0.271	0.522 ±0.065				

* *Ecart type.*

nous avons opéré, la baisse du cholestérol des surrénales, qui n'était pas significative chez les témoins non traités exposés au froid pendant 48 heures, le devient au bout de 72 heures; autrement dit, les résultats obtenus par Long (8) sont confirmés, mais avec un décalage de plus de deux jours, décalage imputable au fait que la température employée dans notre cas était plus élevée ou que les rats employés dans l'expérience de Long étaient plus petits. Le décalage ainsi obtenu nous a permis d'amplifier le phénomène, et surtout d'observer que les rats traités à l'acide ascorbique montraient cette baisse caractéristique du cholestérol surrénalien bien avant les témoins non traités. Quant aux témoins traités au bicarbonate, véritables témoins ceux-là, la diminution du cholestérol dans les surrénales bien qu'assez importante n'est pas encore significative au bout de ces trois jours d'exposition au froid.

La fig. 1 est une représentation graphique des trois tableaux précédents:

(a) Cholestérol %: il n'y a, chez le groupe témoin, aucune baisse significative durant les trois premiers jours d'exposition au froid. Chez le groupe qui reçoit de l'acide ascorbique cette réduction est très prononcée et toujours significative. Chez le groupe qui ne reçoit rien il y a une réduction significative du cholestérol seulement après 72 heures d'exposition au froid.

(b) Cholestérol total: chez le groupe traité à l'acide ascorbique il y a une baisse significative du cholestérol total après 24 et 72 heures d'exposition. Dans aucun autre cas constatons-nous une baisse significative.

Le Tableau VII montre les résultats obtenus chez le cobaye. Des animaux pesant environ 300 grammes ont été exposés au froid durant 24 heures. Chez le groupe qui reçoit le plus d'acide ascorbique, le cholestérol a diminué dans les surrénales, tandis que chez le groupe qui reçoit environ 10 fois moins d'acide ascorbique par jour, le cholestérol n'a pas changé par rapport aux valeurs obtenues à température normale.

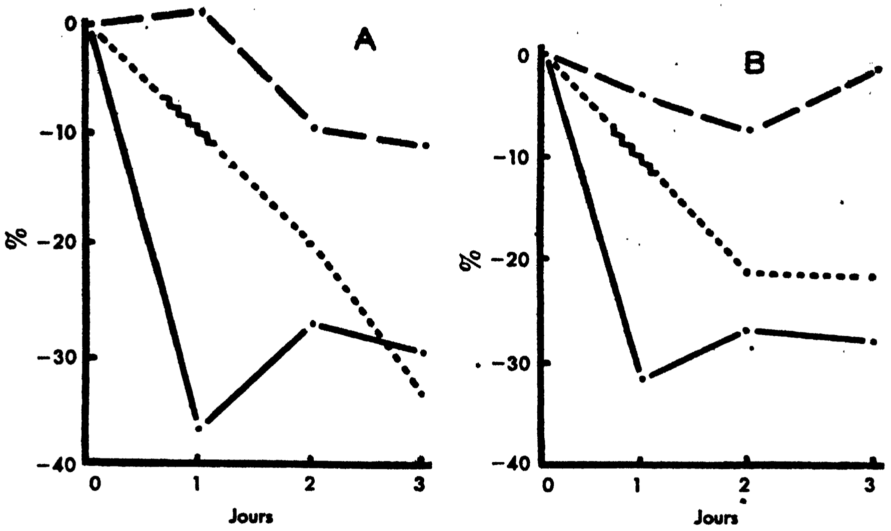

FIG. 1 (*a*) FIG. 1 (*b*)

FIG. 1. *A = cholestérol, %. β = cholestérol total.* ———— *Ascorbate.* —— —— *Témoins.* — — — — — — *Non traités. En ordonnées: pourcentages de variation par rapport aux animaux semblablement traités, à la température normale.*

TABLEAU VII

	Nombre d'animaux	Groupe II 25 mg./jour			Groupe IX 2 mg./jour		
		Température normale	Froid	Différence, %	Température normale	Froid	Différence, %
Cholestérol, %	27	6.34	5.08	−19.87	4.65	4.77	+2.52
Cholestérol total	27	4.22	3.45	−18.25	3.68	3.66	−0.55

On obtient donc ici des résultats analogues à ceux que nous avons obtenus chez le rat, pour le même temps d'exposition au froid.

Ces résultats indiquent que l'acide ascorbique, loin d'empêcher l'activité de la surrénale pendant l'exposition au froid, semble au contraire la favoriser, si la baisse du cholestérol est vraiment un indice sûr de cette activité.

(4) *Pression Systolique*

La méthode que nous avons employée pour mesurer la pression des animaux en expérience est celle du pléthysmographe (William, Harrison et

Grollman (15)) modifiée par Friedman (5). On sait que ce dernier, pour des raisons justifiées et indiscutables, a éliminé la nécessité de chauffer l'appareil et l'animal au moment des déterminations. On comprend qu'une telle modification s'adaptait merveilleusement à nos protocoles expérimentaux où les rats étaient soumis au froid et ne devaient en être sortis que pendant quelques minutes (un maximum de dix minutes) pour les besoins des déterminations.

Comme on peut le constater, d'après le Tableau VIII, la pression systolique d'un groupe de rats gardés à température normale et ayant reçu pendant 48 heures, 150 mg. d'acide ascorbique par jour (sous forme d'ascorbate de sodium) par injections intrapéritonéales, est de 19.7 mm. Hg moins élevée (baisse hautement significative) que la pression initiale observée avant le traitement à l'ascorbate de sodium. Chez les animaux du même groupe et exposés au froid pendant les deux jours de traitement, la pression subit une baisse équivalente là encore hautement significative.

TABLEAU VIII

EXPÉRIENCE DE 48 HEURES

Poids initial	Poids final	Pression initiale	Pression finale	Diff.	*t*	Traitements
Diff. entre ascorbate temp. normale et ascorbate froid: −2.6 (t = 0.7)						
202.5	203.3	107.6 ± 1.0* (131)**	87.9 ± 1.9 (22)	−19.7	*9.3*	Ascorbate de sodium Température normale
198.6	188.8	107.6 ± 1.0 (131)	85.3 ± 3.2 (17)	−22.3	*6.7*	Ascorbate de sodium Froid
Diff. entre bicarbonate temp. normale et bicarbonate froid: −23.0 (t = 8.2)						
199.3	200.8	107.6 ± 1.0 (131)	103.2 ± 1.8 (21)	− 4.4	2.2	Bicarbonate de sodium Température normale
203.7	192.6	107.6 ± 1.0 (131)	80.2 ± 2.2 (16)	−27.4	*11.4*	Bicarbonate de sodium Froid
Diff. entre témoins temp. normale et témoins froid: −33.5 (t = 5.7)						
192.6	191.0	107.6 ± 1.0 (131)	107.5 ± 2.6 (10)	− 0.1	0.0	Témoins Température normale
196.4	181.0	107.6 ± 1.0 (131)	74 ± 5.4 (9)	−33.6	*6.1*	Témoins Froid

* *Ecart type.*

** *Nombre d'animaux.*

N.B. σm *est calculé d'après* ($N = N - 1$) *sauf pour les témoins où* $N = N - 2$.

Par contre, chez les rats traités au bicarbonate de sodium pendant le même temps, la pression systolique qui ne diminue que de 4.4 mm. Hg à température normale, subit une chute très marquée lorsque ces animaux sont exposés au froid. On observe le même phénomène chez les témoins ne recevant aucune injection: la pression demeure à son niveau normal à température ordinaire, tandis qu'au froid elle baisse de façon dramatique.

En somme l'hypotension due au froid, observée chez les rats témoins non traités ainsi que chez les animaux recevant des injections de bicarbonate de sodium, ne semble pas se produire chez les rats traités à l'acide ascorbique, puisque la baisse de pression obtenue chez les rats recevant de l'ascorbate de sodium au froid n'est pas plus prononcée que celle obtenue chez les rats traités de la même façon mais gardés à température normale. La différence entre la pression des rats recevant de l'ascorbate au froid et celle des animaux du même groupe gardés à température ordinaire n'est pas significative tandis qu'elle l'est chez les témoins non traités et les témoins recevant du bicarbonate. (Les différences entre les divers groupes, au froid, ne sont pas significatives. Voir expérience de 72 heures.)

Sur six autres groupes d'animaux de même poids, nous avons répété la même expérience mais en la prolongeant d'une journée (soit trois jours: Tableau IX).

Comme le montre le Tableau IX, après trois jours de traitement, l'ascorbate n'a plus d'effet sur la pression des rats maintenus à température normale; au froid, la baisse de pression qui était de 22.3 mm. Hg après 48 heures n'est plus que de 8.5 mm. Hg après 72 heures.

Chez les traités au bicarbonate comme chez les témoins non traités, la pression demeure normale à la température du laboratoire, tandis qu'elle baisse au froid, un peu moins toutefois qu'après 48 heures.

Si l'on compare les groupes au froid par rapport à leurs témoins à température normale, on constate que chez les traités à l'ascorbate, la pression ne baisse que de 8 mm. de Hg (baisse peu significative) alors que chez les traités au bicarbonate elle baisse de 17.7 mm. Hg; finalement chez les non traités elle baisse de 26 mm. Hg. (Les deux derniers résultats étant hautement significatifs, de même que les différences entre les divers groupes au froid.)

En résumé, comme en fait foi la figure 2, l'ascorbate de sodium semble prévenir l'hypotension caractéristique du shock; en effet, si l'on exprime en pourcentage les variations de la pression systolique chez les rats exposés au froid par rapport à celle des témoins gardés à température normale, on constate que chez les traités à l'ascorbate, la pression systolique varie très peu tandis que chez les traités au bicarbonate et chez les témoins non traités il se produit une baisse de pression marquée.

Acide Ascorbique et Hypertension

Dans une troisième expérience, nous avons cherché à savoir si l'acide ascorbique pouvait abaisser la pression des animaux rendus hypertensifs par une exposition prolongée à un stress tel qu'un froid de −2 à 0° C.

TABLEAU IX

EXPÉRIENCE DE 72 HEURES

Poids initial, grs	Poids final, grs	Pression initiale	Pression finale	Diff.	*t*	Traitement
Diff. entre ascorbate temp. normale et ascorbate froid: 8.0 (t = 2.5)						
213.8	217.8	107.6 ± 1.0* (131)**	107.1 ± 2.0 (24)	− 0.5	0.2	Ascorbate de sodium Température normale
213.6	201.0	107.6 ± 1.0 (131)	99.1 ± 2.4 (24)	− 8.5	*3.2*	Ascorbate de sodium Froid
Diff. entre bicarbonate temp. normale et bicarbonate froid: −17.7 (t = 4.4)						
211.3	216.3	107.6 ± 1.0 (131)	109.6 ± 1.8 (27)	+ 2.0	1.0	Bicarbonate de sodium Température normale
213.0	199.4	107.6 ± 1.0 (131)	91.9 ± 3.6 (20)	−15.7	*4.7*	Bicarbonate de sodium Froid
Diff. entre témoins temp. normale et témoins froid: −26.0 (t = 10.0)						
213.5	222.7	107.6 ± 1.0 (131)	112.6 ± 0.6 (23)	+ 5.0	*4.5*	Témoins Température normale
213.2	203.9	107.6 ± 1.0 (131)	86.6 ± 2.6 (23)	−21.0	*7.7*	Témoins Froid

* *Ecart type.*
** *Nombre d'animaux.*

Nous avons donc placé dans une chambre froide, 32 rats pesant en moyenne 295 grammes; 16 de ces rats recevaient une solution de NaCl à 1% ad libitum, tandis que les 16 autres recevaient de l'eau de boisson. Ces deux groupes avaient évidemment leurs témoins à température normale.

Au début, la pression normale de ces 64 rats était de 87.7 mm. de Hg.

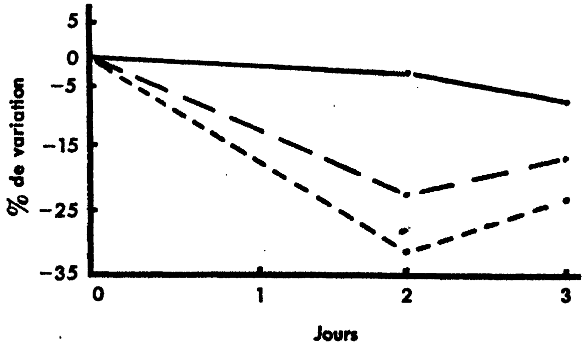

FIG. 2. *Variations de la pression systolique chez des rats exposés au froid par rapport à leurs témoins à la température normale.*

Après un mois d'exposition au froid, voici quelle était la pression moyenne de chaque groupe de rats (Tableau X):

TABLEAU X

	Température normale		Froid			
	Pression initiale	Pression après 1 mois	Pression initiale	Pression après 1 mois	Diff.	*t*
Non traités	88.7 ± 3.3* (16)	85 ± 3.4 (16)	90.6 ± 2.2 (16)	108 ± 6.6 (16)	+17.4	2.5
NaCl 1%	86.3 ± 3.1 (16)	84.4 ± 3.0 (16)	85.8 ± 5.5 (16)	115 ± 7.7 (16)	+29.2	2.2

* *Ecart type.*

Au froid, parmi les 16 non traités quatre manifestaient une augmentation de pression d'environ 58%, tandis que parmi les 16 buvant du NaCl à 1%, six montraient une augmentation de pression d'environ 54%.

Nous avons alors partagé ces 10 rats en deux groupes de cinq: au premier groupe nous avons donné 150 mg. d'acide ascorbique en trois injections intrapéritonéales de 50 mg. chacune à toute les quatre heures; au deuxième groupe nous avons injecté du bicarbonate de sodium suivant le même protocole.

Comme le montre la figure 3, dès une heure après la première injection, la pression moyenne des cinq rats ayant reçu de l'acide ascorbique baisse de

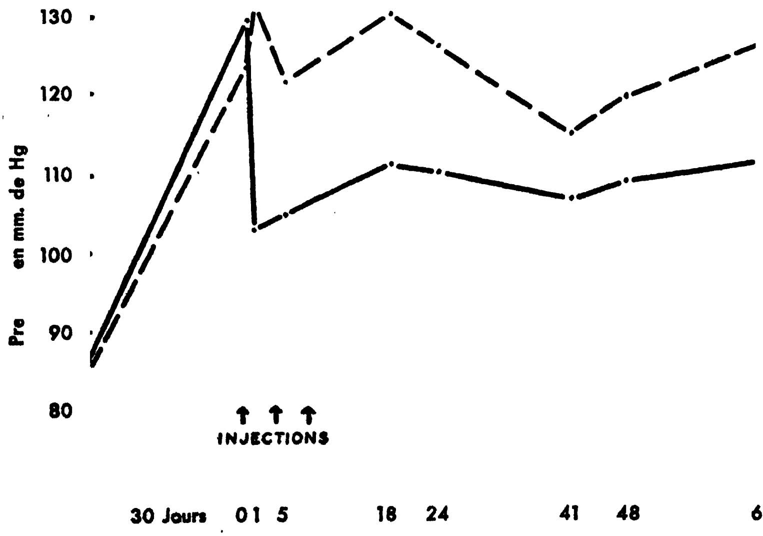

FIG. 3. *Effets de l'acide ascorbique sur la pression systolique au froid (de −2° à 0° C.)*
———— *Ascorbate.* — — — *Bicarbonate.*

130 mm. de Hg à 103 mm. Hg soit une chute de 26% tandis que ceux recevant du bicarbonate voient leur pression monter de 124 à 132 mm. de Hg, soit une élévation d'environ 7%.

On peut également constater que 65 heures après la première injection, l'effet des 150 mg. d'acide ascorbique se fait encore sentir alors que la pression des témoins ayant reçu du bicarbonate se maintient toujours élevée.

Après trois jours et neuf heures la pression moyenne des cinq rats traités à l'acide ascorbique étant de 114 mm. Hg nous avons alors donné à ces animaux une nouvelle injection de 50 mg. d'acide ascorbique et, une heure après, nous constations que la pression était descendue à 82 mm. de Hg soit la pression enregistrée au début de l'expérience.

Cette dernière expérience, que nous considérons comme préliminaire, étant donné le nombre restreint d'animaux employés, semble cependant indiquer que l'acide ascorbique abaisse l'hypertension provoquée par une exposition prolongée à un froid de −2° C.

Résumé et Conclusions

Deux de nous ont déjà montré, dans une communication récente, que l'acide ascorbique, à fortes doses, empêche l'hypertrophie de la surrénale chez le rat et le cobaye exposés au froid, tout en augmentant leur résistance aux mêmes conditions défavorables (4); cette observation a entraîné toute une série de recherches connexes destinées à faire connaître les effets de l'acide ascorbique, dans les conditions déjà précisées, sur les phénomènes physiologiques et pathologiques normalement associés à l'hypertrophie de la surrénale. C'est ainsi qu'on a pu constater, qu'au froid, l'acide ascorbique:

(1) inhibe partiellement l'atrophie du thymus qui est très marquée chez les témoins;

(2) accélère l'apparition de l'hypertrophie de la thyroïde, alors que les témoins montrent au même moment l'atrophie qu'on rencontre habituellement aux premiers stages de l'exposition au froid;

(3) cause une augmentation du poids de la rate, contrairement à ce qui arrive chez les témoins qui montrent une diminution;

(4) diminue la concentration d'histamine dans les surrénales et les reins:

(5) cause une diminution plus rapide du cholestérol des surrénales;

(6) diminue de façon très significative l'hypotension caractéristique des premières heures ou des premiers jours (suivant les conditions) d'exposition au froid.

Ainsi, en tenant compte des résultats antérieurs obtenus sur les surrénales et des résultats du présent mémoire, traitant des variables normalement associées à l'hypertrophie de la surrénale, nous pouvons considérer que la physiologie des animaux exposés au froid semble adaptée d'avance, grâce à l'acide ascorbique, aux exigences imposées par leur nouveau mode de vie:

le "stress" normalement causé par le froid diminue grandement et est même complètement aboli, dans certains cas, par l'acide ascorbique administré à doses massives.

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L'ACTION ANÉMIANTE DE DEUX ANTAGONISTES DE LA LYSINE: L'HEXAHOMOSÉRINE ET L'ACIDE α-AMINOADIPIQUE[1]

EDOUARD PAGÉ, ROSAIRE GINGRAS, ET ROGER GAUDRY

Abstract

The addition of hexahomoserine (α-amino-ε-hydroxycaproic acid) to complete rations produces growth inhibition, anemia, and a considerable rise in blood plasma amino nitrogen in the rat. Simultaneous administration of lysine prevents the anemia and partially restores growth but does not affect the amino nitrogen level in the plasma. These effects of hexahomoserine and the antagonistic action of lysine are even more striking in animals previously depleted of lysine. Aminoadipic acid cannot replace lysine in the ration and it produces anemia in lysine-depleted rats. Its action is also counteracted by lysine. Liver concentrates and folic acid are ineffective in the prevention of the anemia caused by hexahomoserine.

Introduction

Ces expériences portent sur deux composés présentant avec la lysine une parenté chimique étroite: l'acide α-amino-ε-hydroxycaproique (hexahomosérine) et l'acide α-aminoadipique. Nous avons déjà rapporté que l'hexahomosérine ne peut remplacer la lysine chez le rat (5) et que l'addition de ce composé à une ration complète provoque l'anémie chez ce même animal (7). Nous avons plus tard constaté que l'acide aminoadipique ne peut remplacer la lysine chez le rat (8) et ceci vient d'être confirmé par Geiger et Dunn (3).

Borsook et ses collaborateurs (1) avaient antérieurement démontré que l'acide aminoadipique est un dérivé biologique de la lysine. Enfin, l'on sait depuis les travaux de Osborne et Mendel (6), en 1914, que la lysine est un acide aminé essentiel et Gillespie, Neuberger et Webster (4) ont observé en 1945 qu'une carence prolongée en ce composé provoque l'anémie chez le rat.

Protocole Expérimental et Méthodes

La ration de base utilisée est celle qui est indiquée au Tableau I, sauf lorsque les rats furent soumis à un régime dépourvu de lysine. La caséine fut alors remplacée par 17.4% de zéine et 0.6% de DL-tryptophane. L'addition d'acides aminés ou de foie fut faite aux dépens du saccharose.

Les sels minéraux étaient constitués du mélange U.S.P. XII, No. 2, auquel nous avions ajouté les sels suivants: sulfate de cuivre pentahydraté, 0.3; sulphate de manganèse tétrahydraté, 5.0; iodure de potassium, 0.7; et chlorure de zinc, 0.2 gramme par kilogramme du mélange. Les rats reçurent en plus, par voie orale, un supplément hebdomadaire de vitamines A, D et E.

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[1] *Manuscrit reçu le 20 juillet 1949.*

Contribution du Département de Biochimie, Faculté de Médecine, Université Laval, Québec.

TABLEAU I

COMPOSITION DU RÉGIME DE BASE

(par 100 grammes de ration)

	Groupe témoin
Caséine purifiée (Vitamin test, GBI)	18 g.
Cellulose (Cellu-flour)	2 "
Sels minéraux	4 "
Huile Mazola	5 "
Saccharose	71 "
Chlorhydrate de thiamine	0.4 mg.
Riboflavine	0.5 "
Chlorhydrate de pyridoxine	0.5 "
Pantothénate de calcium	3.0 "
Acide nicotinique	3.0 "
2-Méthyl-1,4-naphtoquinone	0.1 "
Inosite	10.0 "
Chlorure de choline	150.0 "

L'hémoglobine fut déterminée au moyen du dosage spectrophotométrique de l'hématine acide et l'azote aminé libre du plasma par la méthode colorimétrique de Frame (2). Les prises de sang furent faites par section de la queue pour les énumérations globulaires et la mesure de l'hémoglobine; dans la veine jugulaire, pour le dosage de l'azote aminé.

Des jeunes rats albinos mâles furent utilisés dans tous les cas.

Résultats

(1) *Action anémiante de l'hexahomosérine*

Deux groupes de rats furent utilisés, l'un recevant la ration de base et l'autre, cette même ration additionnée de 0.75% d'hexahomosérine. Au bout de 14 jours, nous obtenions les résultats indiqués au Tableau II. Il est évident que ce composé inhibe la croissance et provoque une anémie.

TABLEAU II

ACTION ANÉMIANTE DE L'HEXAHOMOSÉRINE

(Durée de l'expérience: 14 jours)

	Témoins	Hexahomosérine (0.75%)
Nombre de rats	10	23
Poids initial moyen (g.)	54	53
Gains de poids (g.)	47 ± 3.02 *	14 ± 2.25
Hémoglobine (g./100 ml.), taux final	11.4 ± 0.24	8.9 ± 0.41
Globules rouges (millions/mm³), taux final	8.0 ± 0.46	5.5 ± 0.26

* *Erreur standard.*

(2) *Les vitamines du complexe B et l'hexahomosérine*

Afin de savoir si certaines vitamines du complexe B (autres que celles déjà incluses dans le régime) étaient susceptibles de prévenir cette anémie, nous avons administré l'hexahomosérine en même temps qu'un concentré de foie (Fraction "L"). Trois groupes de rats furent utilisés, le premier recevant une ration de base, le deuxième, cette ration additionnée de 1.5% d'hexahomosérine, et le troisième groupe recevant à la fois 1.5% d'hexahomosérine et 2% de foie. Tel qu'on peut le constater au Tableau III, l'addition de foie ne modifie en rien les effets toxiques de l'hexahomosérine.

TABLEAU III

CONCENTRÉ DE FOIE ET ACTION ANÉMIANTE DE L'HEXAHOMOSÉRINE

(Durée de l'expérience: 14 jours)

	Témoins	Hexahomosérine (1.5%)	Hexahomosérine (1.5%) et foie (2%)
Nombre de rats	10	20	10
Poids initial moyen (g.)	57	57	57
Gains de poids (g.)	63 ± 2.07	20 ± 1.28	19 ± 2.38
Hémoglobine (g./100 ml.)			
Taux initial	9.8 ± 0.34	9.9 ± 0.23	10.1 ± 0.30
Taux final	12.2 ± 0.18	9.3 ± 0.30	9.1 ± 0.31
Globules rouges (millions/mm^3), taux final	6.5 ± 0.17	5.4 ± 0.15	5.5 ± 0.30

A cause de l'importance de l'acide folique dans le traitement de certains types d'anémie, nous avons ensuite réparti les rats du troisième groupe en deux sous-groupes au bout de deux semaines, alors que le concentré de foie s'était déjà avéré inefficace (Tableau III). Nous avons remplacé ce dernier par 2.0 mg. d'acide folique par 100 grammes de ration dans un cas, tandis que l'autre sous-groupe recevait la ration contenant l'hexahomosérine sans supplément vitaminique. Deux semaines plus tard, les rats étaient de plus en plus anémiés et cela, indépendamment de la teneur du régime en acide folique (Tableau IV).

Des résultats de ces deux expériences, il semble logique de conclure que les vitamines du complexe B n'affectent en rien les propriétés toxiques de l'hexahomosérine.

(3) *La lysine et l'hexahomosérine*

Les similarités de structure qui existent entre la lysine et l'hexahomosérine permettaient, par ailleurs, de soupçonner la possibilité d'un antagonisme biologique entre ces deux composés. Il importait donc d'étudier l'effet d'un excès de lysine dans la ration, sur le décours de l'anémie normalement provoquée par l'hexahomosérine. Cependant, nous avons d'abord cru bon de

TABLEAU IV

ACIDE FOLIQUE ET ACTION ANÉMIANTE DE L'HEXAHOMOSÉRINE

(Durée de l'expérience: 15 jours)

	Hexahomosérine (1.5%)	Hexahomosérine (1.5%) et acide folique (2.0 mg. %)
Nombre de rats	5	5
Poids initial moyen (g.)	75	76
Gains de poids (g.)	16 ± 4.35	10 ± 5.38
Hémoglobine (g./100 ml.)		
Taux initial	9.2 ± 0.38	9.0 ± 0.48
Taux final	5.7 ± 1.17	6.0 ± 1.08
Globules rouges (millions/mm^3)		
Taux initial	5.7 ± 0.31	5.3 ± 0.20
Taux final	4.1 ± 0.96	3.6 ± 0.52

nous assurer que l'addition d'un fort pourcentage de DL-lysine au régime ne modifiait pas la formule sanguine et n'avait pas non plus d'effet toxique. A cette fin, deux groupes de rats furent utilisés. L'un reçut la ration de base ordinaire et l'autre, cette même ration additionnée de 4% de DL-lysine. Comme on peut le voir au Tableau V, ce supplément n'affecte ni la croissance, ni les taux sanguins d'hémoglobine et de globules rouges.

TABLEAU V

ADDITION DE DL-LYSINE À UN RÉGIME COMPLET

(Durée de l'expérience: 20 jours)

	Témoins	DL-lysine (4%)
Nombre de rats	5	6
Poids initial moyen (g.)	78	77
Gains de poids (g.)	52 ± 2.77	55 ± 5.00
Hémoglobine, taux final (g./100 ml.)	14.0 ± 0.36	14.6 ± 0.39
Globules rouges, taux final (millions/mm^3)	8.71 ± 0.52	8.55 ± 0.61

L'étape suivante consista à donner à un groupe de rats 1% d'hexahomosérine et 6% de DL-lysine, tandis que deux autres groupes servaient de témoins, l'un étant à la ration de base et l'autre recevant 1% d'hexahomosérine sans supplément. Les résultats de cette expérience sont indiqués au Tableau VI.

TABLEAU VI

LYSINE ET ACTION ANÉMIANTE DE L'HEXAHOMOSÉRINE

(Durée de l'expérience: 54 jours)

	Témoins	Hexahomosérine (1%)	Hexahomosérine (1%) DL-lysine (6%)
Nombre de rats	10	10	10
Poids initial moyen (g.)	53	53	53
Gains de poids (g.)	189 ± 7.73	68 ± 8.28	115 ± 6.18
Hémoglobine (g./100 ml.)			
Taux initial	11.1 ± 0.37	11.2 ± 0.50	11.4 ± 0.31
Taux à 31 jours	13.2 ± 0.44	11.4 ± 0.66	13.5 ± 0.32
Taux à 53 jours	14.6 ± 0.38	12.6 ± 0.47	14.2 ± 0.27
Globules rouges (millions/mm^3)			
Taux initial	6.2 ± 0.10	6.4 ± 0.23	6.2 ± 0.22
Taux à 31 jours	8.3 ± 0.33	6.7 ± 0.40	8.3 ± 0.34
Taux à 53 jours	9.4 ± 0.31	7.1 ± 0.57	8.9 ± 0.44
Volume des globules rouges (%)	—	49 ± 1.34	52 ± 0.91
Azote aminé (mg./100 ml. de plasma), taux final	9.8 ± 0.40	17.6 ± 0.90	20.5 ± 0.70

Il ressort de cette expérience que chez le rat recevant de l'hexahomosérine, l'addition simultanée de DL-lysine prévient l'anémie et rétablit partiellement le taux de croissance à son niveau normal. Il semble de plus que l'hexahomosérine inhibe la croissance non seulement en tant qu'antagoniste de la lysine, mais aussi en se maintenant à un taux élevé dans le milieu intérieur, ce qui doit affecter défavorablement la concentration cellulaire des autres acides aminés.

Il est évident que le supplément de DL-lysine n'abaisse pas la teneur du plasma en azote aminé; ceci n'a rien d'étonnant si l'hexahomosérine, bien que toxique, est réabsorbée au niveau du rein avec la même efficacité que les autres acides α-aminés.

Nous avons de plus confirmé cet antagonisme entre la lysine et l'hexahomosérine chez des rats préalablement carencés en lysine. A cette fin, 40 rats furent soumis durant 43 jours à un régime dépourvu de lysine (régime zéine-tryptophane). Les rats furent ensuite répartis en quatre groupes égaux comme suit: Groupe I: ration de base sans lysine; Groupe II: ration de base et 1% d'hexahomosérine; Groupe III: ration de base, 1% d'hexahomosérine et 1% de *l*-lysine; Groupe IV: ration de base et 1% de *l*-lysine. Quatre des dix rats du Groupe II moururent avant la fin de l'expérience et les résultats indiqués au Tableau VII et à la Fig. 1 ne tiennent compte que des survivants.

TABLEAU VII

EFFET DE LA LYSINE SUR L'ACTION ANÉMIANTE DE L'HEXAHOMOSÉRINE CHEZ LE RAT PRÉALABLEMENT CARENCÉ EN LYSINE

(Durée de l'expérience: 21 jours)

	Ration de base	Hexahomosérine (1%)	Hexahomosérine (1%) lysine (1%)	Lysine (1%)
Nombre de rats	10	6	10	10
Changements de poids (g.)	− 7 ± 0.96	− 14 ± 1.87	+ 14 ± 3.16	+ 42 ± 2.42
Hémoglobine (g./100 ml.)				
Taux inital	11.9 ± 0.18	11.9 ± 0.21	11.9 ± 0.21	11.9 ± 0.18
Taux final	12.2 ± 0.06	7.5 ± 0.94	13.2 ± 0.28	13.8 ± 0.25
Globules rouges (millions/mm^3)				
Taux initial	7.9 ± 0.23	8.1 ± 0.37	7.8 ± 0.28	7.9 ± 0.21
Taux final	7.8 ± 0.20	4.9 ± 0.64	8.5 ± 0.28	9.1 ± 0.37

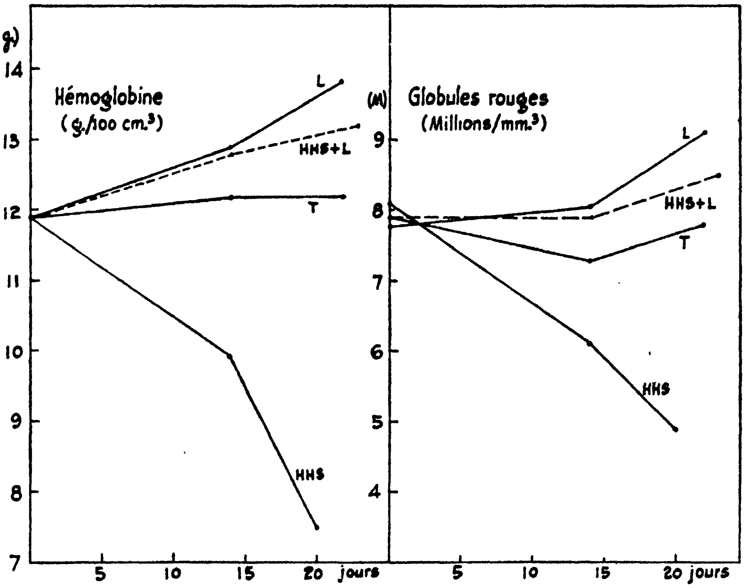

FIG. 1. *Effet de la lysine sur l'action anémiante de l'hexahomosérine chez le rat préalablement carencé en lysine.*

L'analyse statistique révèle qu'il n'existe pas de différence significative entre les deux groupes recevant de la lysine (avec ou sans hexahomosérine) au terme de l'expérience, en ce qui concerne les taux sanguins d'hémoglobine et de globules rouges. Par contre, les rats intoxiqués à l'hexahomosérine (Groupe II) sont très anémiés. Ces résultats nous semblent confirmer l'existence d'un antagonisme entre l'hexahomosérine et la lysine.

(4) *Particularités de l'action anémiante de l'acide aminoadipique*

Ces travaux étaient en cours alors que Borsook et ses collaborateurs (1) annonçaient que l'acide aminoadipique est un dérivé biologique de la lysine. La question se posait à savoir si ce composé est un antagoniste de l'hexahomosérine ou s'il possède lui-même des propriétés anémiantes. Dans une première expérience, l'acide aminoadipique fut simplement ajouté à une ration de base au taux de 1.5% du régime, tandis qu'un autre groupe de rats servait de témoins et qu'un troisième recevait 1.5% d'hexahomosérine dans son régime. Au bout de 16 jours, nous observions les résultats rapportés au Tableau VIII.

TABLEAU VIII

ADDITION D'ACIDE AMINOADIPIQUE À UN RÉGIME COMPLET

(Durée de l'expérience: 16 jours)

	Témoins	Acide aminoadipique (1.5%)	Hexahomosérine (1.5%)
Nombre de rats	6	6	6
Poids initial moyen (g.)	95	93	91
Gains de poids (g.)	64 ± 3.11	53 ± 3.50	5 ± 5.45
Hémoglobine (g./100 ml.)			
Taux initial	10.0 ± 0.34	10.4 ± 0.44	10.9 ± 0.21
Taux final	12.6 ± 0.36	13.0 ± 0.39	10.4 ± 0.51
Globules rouges (millions/mm³)			
Taux initial	6.6 ± 0.52	6.7 ± 0.24	6.7 ± 0.29
Taux final	7.7 ± 0.41	7.8 ± 0.40	6.1 ± 0.31
Azote aminé du plasma (mg./100 ml.)	10.9 ± 0.42	10.6 ± 1.61	21.8 ± 1.20

Dans les conditions de l'expérience, l'acide aminoadipique n'eut aucun effet déprimant sur les taux sanguins d'hémoglobine et de globules rouges. Il semble aussi qu'il ne se produit pas d'accumulation de ce composé dans l'organisme, le taux d'azote aminé du plasma sanguin étant normal. L'acide aminoadipique eut un effet cathartique, ce qui explique peut-être les légères différences observées dans les taux de croissance. Enfin, les rats à l'hexahomosérine sont anémiés, ont un taux élevé d'azote aminé dans le plasma et un taux de croissance à peu près nul, ce qui confirme nos résultats antérieurs.

Il semblait non moins intéressant de rechercher si l'acide aminoadipique exercerait une action antagoniste vis-à-vis de l'hexahomosérine. Un petit nombre d'animaux furent donc carencés en lysine de la façon décrite précédemment. Au bout de 36 jours, les rats furent répartis en quatre groupes comme suit: Groupe I (quatre rats): 1% d'hexahomosérine; Groupe II (quatre rats): 2% d'acide aminoadipique; Groupe III (cinq rats): 1% d'hexahomosérine et 2% d'acide aminoadipique; Groupe IV (cinq rats): 2% de DL-lysine, 2% d'acide aminoadipique et 1% d'hexahomosérine. Ces divers suppléments furent ajoutés à la ration de base dépourvue de lysine. Deux rats du Groupe III moururent en cours d'expérience. Les résultats obtenus sont illustrés à la Fig. 2. Notons tout d'abord que l'acide aminoadipique ne peut remplacer la lysine chez le rat et n'est pas non plus un antagoniste de l'hexahomosérine. Bien plus, ce composé semble exercer une action anémiante en tous points comparable à celle de l'hexahomosérine. Enfin, la lysine semble antagoniser à la fois ces deux composés.

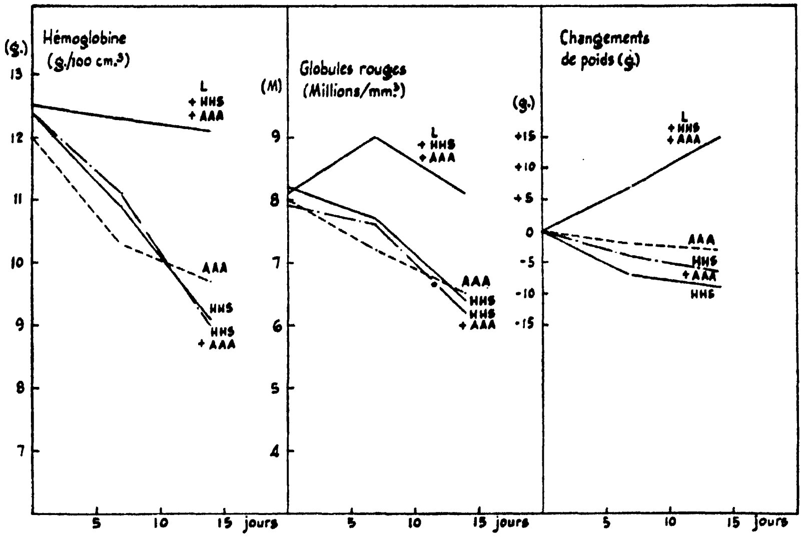

FIG. 2. *Action anémiante de l'acide α-aminoadipique (au taux de 2%) chez le rat préalablement carencé en lysine et effet antagoniste de la lysine.*

Nous avons donc repris cette expérience avec un plus grand nombre de rats et en ayant soin, cette fois, d'inclure un groupe témoin à la ration de base (sans lysine). La période de carence en lysine fut de 41 jours. Quatre groupes furent alors composés comme suit: Groupe I: ration de base (sans lysine); Groupe II: ration de base et 1% d'acide aminoadipique; Groupe III: ration de base et 1% d'hexahomosérine; Groupe IV: ration de base, 2% de DL-lysine et 1% d'acide aminoadipique. Un rat du Groupe II et cinq rats du Groupe III moururent en cours d'expérience. La Fig. 3 illustre les changements progressifs dans les taux d'hémoglobine et le nombre de globules rouges de ces quatre groupes. On trouvera au Tableau IX les valeurs finales pour ces mêmes groupes.

Le groupe à l'acide aminoadipique est plus anémié que celui simplement dépourvu de lysine (témoins) et les différences sont significatives en ce qui concerne le taux d'hémoglobine et le volume des globules rouges (t = 2.50 et 3.23 respectivement), mais non quant au nombre de globules rouges (t = 1.74). Il est clair que les rats recevant l'hexahomosérine sont beaucoup plus anémiés. Cette fois-ci, l'action anémiante de l'acide aminoadipique n'est pas superposable à celle de l'hexahomosérine, mais il faut noter que la ration ne contenait que 1% d'acide aminoadipique comparativement à 2% au cours de l'expérience précédente. Enfin, la lysine prévient encore une fois les effets anémiants de l'acide aminoadipique.

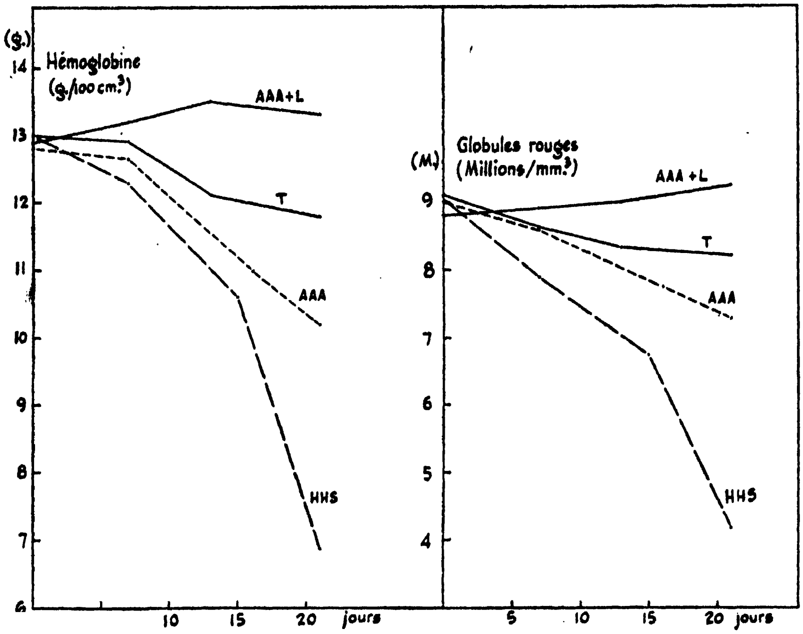

FIG. 3. *Action anémiante de l'acide α-aminoadipique (au taux de 1%) chez le rat préalablement carencé en lysine et effet antagoniste de la lysine.*

TABLEAU IX

LYSINE ET ACTION ANÉMIANTE DE L'ACIDE AMINOADIPIQUE ET DE L'HEXAHOMOSÉRINE CHEZ LE RAT PRÉALABLEMENT CARENCÉ EN LYSINE

(Durée de l'expérience: 21 jours)

	Témoins	Acide aminoadipique (1%)	Hexahomo-sérine (1%)	DL-lysine (2%), acide AAA (1%)
Nombre initial de rats	8	9	8	9
Nombre de survivants	8	8	3	9
Hémoglobine (g./100 ml.), taux final	11.8 ± 0.47	10.2 ± 0.41	6.9	13.3 ± 0.44
Globules rouges (millions/mm³), taux final	8.2 ± 0.27	7.3 ± 0.45	4.2	9.3 ± 0.28
Volume des globules rouges (%), taux final	51 ± 1.48	43 ± 2.03	28	53 ± 1.36

Discussion et Conclusions

Il est intéressant de noter que l'acide aminoadipique n'est pas anémiant lorsque la ration contient une quantité adéquate de lysine (18% de caséine dans la ration); ce composé a peu d'effet chez le rat carencé en lysine lorsqu'on l'inclut au taux de 1% du régime, mais son action est très nette dans les mêmes conditions expérimentales au taux de 2%. Nous attribuons ceci au fait que *l'acide aminoadipique ne s'accumule pas dans le sang* (et les tissus) *comme il arrive dans le cas de l'hexahomosérine.* Quant à ce dernier composé, il est évident qu'à cause de sa rétention dans le sang, *le rapport lysine: hexahomosérine des tissus est bien différent de ce qu'il est dans la ration.* L'index d'inhibition tel qu'observé dans le cas des microorganismes, et basé sur la composition du mélange nutritif, ne saurait donc servir de critère du phénomène de l'antagonisme chez le mammifère si l'antagoniste (e.g. l'hexahomosérine) est réabsorbé au niveau du rein et s'accumule dans le milieu intérieur alors que l'excès du composé naturel est constamment catabolisé.

Les conclusions suivantes semblent donc s'imposer:

(1) Dans les conditions expérimentales décrites plus haut, l'hexahomosérine et l'acide aminoadipique provoquent l'anémie chez le rat. Dans le cas de l'acide aminoadipique, cependant, l'anémie ne se produit que lorsque l'animal a été préalablement carencé en lysine.

(2) L'addition d'un supplément de lysine à la ration prévient l'apparition de ces anémies.

(3) Comme au surplus, d'autres auteurs ont démontré que la carence prolongée en lysine entraîne elle aussi une anémie, il est probable que les deux composés sont de véritables antagonistes de la lysine.

(4) En plus de son action anémiante et anti-lysine, l'hexahomosérine semble être toxique du simple fait de son accumulation dans le milieu intérieur. Peut-être la concentration cellulaire des autres acides aminés en est-elle affectée défavorablement.

Remerciements

Les auteurs sont heureux de remercier le Conseil National des Recherches pour son aide matérielle.

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EFFECTS OF ESERINE AND ACETYLCHOLINE ON THE RESPIRATORY CENTERS AND HYPOGLOSSAL NUCLEI[1]

By Frederick R. Miller

Abstract

The floor of the fourth ventricle is exposed in the cat, decerebrated with the Sherrington Decerebrator. Eserine, 0.15 mgm. per kgm. body weight intravenously, induces extra respirations and tongue retractions, the latter at the same rate as, and slightly preceding, the "original" respirations. Tongue retractions are caused by irradiation from inspiratory centers to hypoglossal nuclei. Acetylcholine (ACh) concentrations of 1 : 5 × 10^7 to 1 : 10^6, applied in a small rectangle of spot test paper to the floor of fourth ventricle, enhance respiratory and lingual effects; these concentrations of ACh or, occasionally, up to 1 : 5 × 10^4 often induce convulsive discharges from hypoglossal nuclei, as shown by powerful tongue contractions. Effects of eserine on respiration are not due to concurrent blood pressure changes, which are insignificant. Intravenous eserine and medullary ACh are believed to stimulate and facilitate various central synapses; both drugs stimulate the inspiratory centers. Medullary ACh excites particularly the eserinized synapses surrounding the motoneurones of the hypoglossal nuclei, thus inducing the convulsive discharges from these nuclei. Effects of eserine and ACh are abolished and precluded by intravenous atropine, results that are in harmony with those of several recent investigators.

Introduction

When acetylcholine (ACh) in extremely low concentrations is applied to the floor of the fourth ventricle of a decerebrated, eserinized cat, there results marked stimulation of the respiratory centers and hypoglossal nuclei. The concentrations of ACh employed varied from 1 : 5 × 10^7 to 1 : 10^6 and the applications were made in small rectangles of spot test paper soaked in ACh dissolved in Ringer–Locke solution made up without sodium bicarbonate. The respirations are accelerated and the tongue shows first furrowing and then retraction, corresponding to each inspiration; later the tongue exhibits violent and extensive contractions, which are not related to the respirations. The violent contractions are the consequence of convulsive discharges from the XII nuclei and it was inferred that medullary ACh stimulates the synaptic endings surrounding the motoneurones of these nuclei (Miller (11)).

Respiratory changes and lingual contractions were recorded graphically and the results, with conclusions, formed the basis of several preliminary reports (Miller (12, 13, 14, 15)). The present paper furnishes a detailed account of these experimental findings, together with their theoretical interpretations.

Methods

The cat was anesthetized with chloroform and ether; tracheotomy was performed and a cannula was tied into a vein, usually the external jugular,

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[1] *Manuscript received July 20, 1949.*

Contribution from the Department of Neurophysiology, University of Western Ontario, London, Ont.

occasionally the femoral; both carotids were ligated. Decerebration under deep anesthesia was by the Sherrington Decerebrator (20), the plane of midbrain transection lying sufficiently caudal to avoid the inconvenience of spontaneous limb movements; the median part of the cerebellum was then excised so as to expose the floor of the fourth ventricle.

After recovery the animal was placed with thorax down on a special table. The flange of skull bone in front of the tentorium was fixed by screws in a semicircle of thick brass; this in turn was held at a suitable angle in the jaws of a heavy brass U, provided at its convex middle with a projecting rod; this latter was clamped to a stout, upright rod, secured to the table top. Thus the skull was held rigid and the medullary floor could be viewed from above, any exuding fluid being removed with small pledgets of absorbent cotton, prior to application of the ACh; this was applied in small rectangles of spot test paper, 2 mm. × 7 mm., pointed at one end, so as to fit the calamus scriptorius and cover the hypoglossal nuclei of both sides; the rectangles were soaked in ACh of the concentrations used and were applied carefully with fine forceps to the medullary floor. The Ringer–Locke solution used to dissolve the ACh was made up without sodium bicarbonate.

To permit recording of the respirations the body was laid with one flank resting on the table top; limbs and tail were secured with string loops. Respirations were recorded by the pneumograph devised by Sherrington (16), a small serrefine on a swivel replacing the needle points to grip the skin of the chest at about the level of the 11th rib. The short arm of the pneumograph lever was connected by a thread to a crank lever, pulling against a light, coiled spring. With these arrangements inspiration was registered as an upward movement on the drum.

To record the lingual movements, the tongue, which was fully exposed in this preparation, was drawn forwards and supported in the horizontal position by a small canvas sling, suspended by threads from a rod above; a small hook, pushed through the tongue tip, was connected by a thread to a crank lever like that used for the respirations. Retractions of the tongue were thus recorded as upward movements of the writing point.

The floor of the fourth ventricle, as exposed in the above procedures, is shown in Fig. 1. The elongated hypoglossal nucleus lies just beneath the surface and very slightly lateral to the shallow groove, one on each side of the midline. This groove served as a useful landmark for exploration of the XII nucleus by unipolar faradization; the results are shown on the left side of Fig. 1. The close proximity of XII nucleus to medullary floor is shown in Fig. 11; thus the nucleus is accessible for direct stimulation by the ACh rectangles.

Results

Tongue Retractions Before the Use of Eserine and Acetylcholine

After intravenous eserine and, more especially, after this drug and medullary ACh the tongue showed retraction with furrowing of its base; the reaction preceded slightly each inspiration. It was important to determine whether the reaction occurred, even to a slight extent, before the use of the drugs.

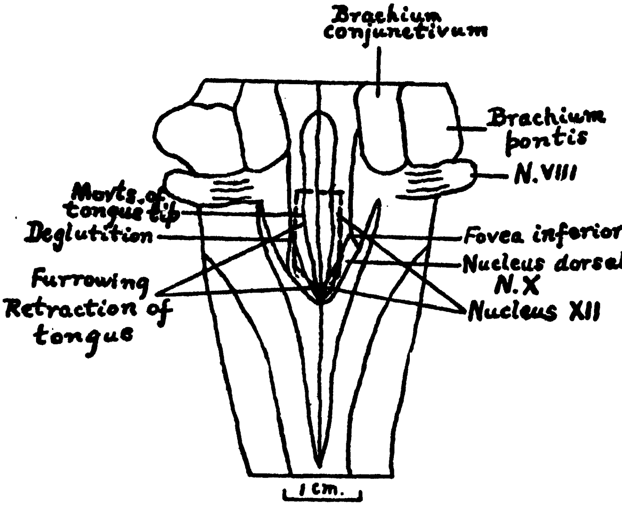

FIG. 1. *Floor of fourth ventricle of cat.* × *2. Location of elongated XII nucleus is shown on right side, just lateral to shallow groove, marking limits of fasciculus longitudinalis medialis. Unipolar faradization along groove impinged on XII nucleus and yielded tongue responses shown on left. Faradization of fovea inferior yielded deglutition, probably from afferent taste and visceral fibers. Rectangles of spot test paper, caudal ends pointed, soaked in low concentrations of ACh, were applied over area enclosed by interrupted lines and yielded results in following experiments. XII nucleus lies just below surface (Fig. 11) and is accessible for direct stimulation by ACh rectangles.*

Observations showed that this tongue reaction actually occurred at various stages of the preparation: sometimes, after tying the carotids, a tongue furrow occurred just prior to each inspiration; a similar effect was noted occasionally after decerebration and also after subsequent decerebellation. Sometimes, after decerebellation, tongue retraction was quite marked; it preceded slightly the corresponding inspiration and was caused partly by the furrow and partly by a generalized contraction of the tongue. Since the lingual furrow was accompanied by simultaneous elevation of the soft palate and dilatation of the faucial pillars it was clearly part of a reaction to facilitate the indrawing of air. Certain of the tongue muscles must, therefore, be regarded as accessory to those of respiration, a familiar example being afforded by the lingual movements of the panting dog; these tongue muscles are thus in the same category as those of the face, especially the *dilatores naris.* In consequence, potential synaptic pathways must be regarded as existing between the inspiratory centers and the hypoglossal nuclei. The slight precedence of the tongue retraction over the corresponding inspiration, just mentioned, is intelligible in view of the shorter conduction pathways from inspiratory centers to XII nuclei to tongue muscles, as compared to those from inspiratory centers to spinal motoneurones to intercostal muscles. The nature of the pathways concerned will be considered more fully under "Discussion".

PLATE I

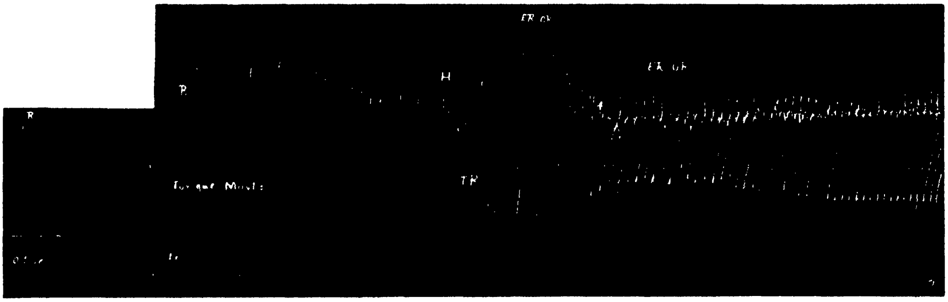

FIG. 2. *In all figures except Fig. 7 upper record, respirations, R; lower record, tongue movements. TR, tongue retractions. At signal 0.5 mgm. eserine into femoral vein. H, humps. ER, extra respirations. OR, original respirations. Letters have same meanings in all figures. Explanations of all figures in text. Time in all figures in 0.5 sec.*

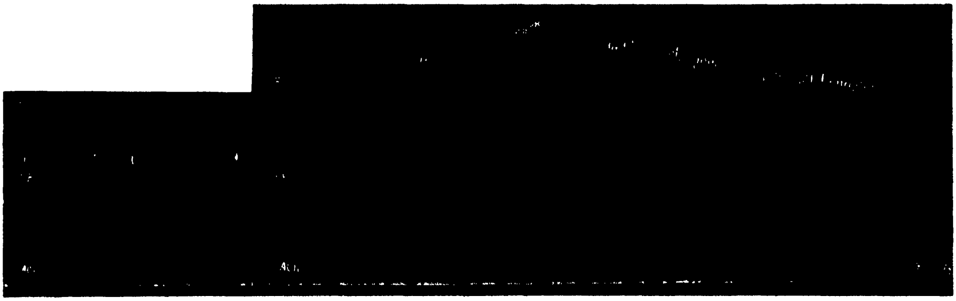

FIG. 3. *Just before this record a rectangle of spot test paper soaked in ACh 1 : 10^7 was applied to floor of fourth ventricle. At first signal ACh 1 : 5 × 10^7 was similarly applied. TRs slightly augmented. At second signal this application was repeated. TRs greatly augmented. At last signal ACh rectangle removed.*

PLATE II

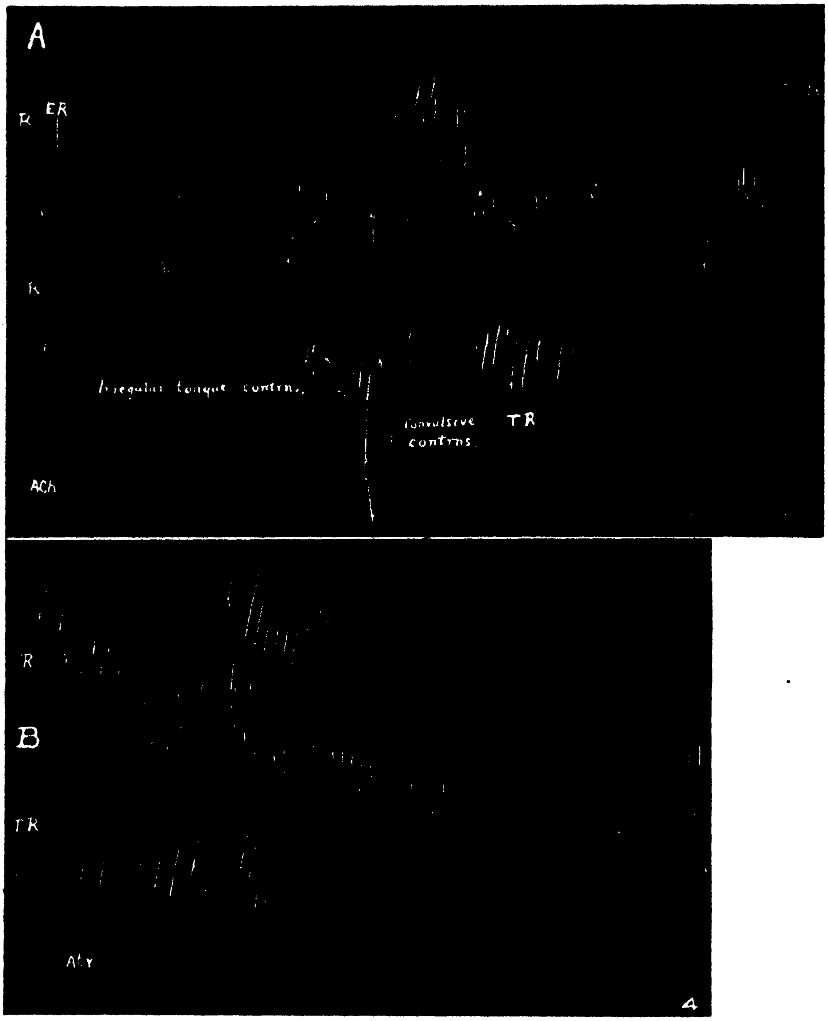

FIG. 4. *A. Just before this record 0.5 mgm. eserine intravenously. At signal rectangle with ACh 1 : 5 × 10⁴ on medullary floor: tongue contractions. B. At signal 3 mgm. atropine intravenously: tongue contractions abolished.*

Description of Records

The following symbols are employed for brevity in descriptions of the records:

D(s):	deglutition(s);
ER(s):	extra respiration(s);
H(s):	"hump(s)" on respiration (s);
OR(s):	original respiration(s);
R(s):	respiration(s);
TR(s):	tongue retraction(s).

The general effects of eserine and ACh on the respiratory centers and hypoglossal nuclei will now be described. Figs. 2 to 4 illustrate the course of a typical experiment. The injection of 0.5 mgm. eserine into the femoral vein (Fig. 2) caused a quickening of the respirations and, in one minute, resulted in the appearance, at the beginning of inspiration, of a small "hump" (H); the humps then developed into extra respirations (ERs), which quickly came to exceed the original respirations (ORs) in amplitude; these latter declined for a time but became of briefer period than before eserine. Immediately after the appearance of the humps, tongue retractions (TRs) developed and rapidly augmented; each TR preceded slightly an original respiration (OR) and owed its production to the OR, that is, to the inducing discharge from the inspiratory center; this will be explained later.

The ERs then declined to small waves; at the same time the ORs enlarged, while their periods remained brief; each TR still preceded slightly an OR (Fig. 2); this relationship was easily proved by inspection of the pneumograph and tongue levers. A spot test paper rectangle soaked in ACh of concentration $1 : 10^7$ applied to the floor of the fourth ventricle then increased further the rate and amplitude of the Rs and likewise those of the TRs, which continued at exactly the same rate as the Rs (Fig. 3). A rectangle with ACh $1 : 5 \times 10^7$ further quickened and augmented both Rs and TR's (Fig. 3), while a second application of ACh of the same concentration had the pronounced effect shown in Fig. 3: here in 29 sec. after the ACh application, the Hs reappeared, developing into ERs; the ERs increased, while the ORs became more rapid and larger. Closely corresponding to the appearance of the ERs the TRs augmented and continued at the quickened rate of the ORs. These effects of medullary ACh thus resembled, in a general way, those of intravenous eserine alone.

The respiratory changes and TRs then subsided, the TRs disappearing first; slight TRs were, however, still visible, though not recorded by the lever; as before each corresponded to a respiration (Fig. 3). After subsidence of the TRs, ACh of concentrations from $1 : 5 \times 10^7$ to $1 : 10^6$ failed to restore them. The TRs were, however, promptly restored by 0.5 mgm. eserine intravenously, while concurrently the ERs recurred. The medullary application of ACh $1 : 5 \times 10^4$ now induced irregular contractions of the tongue

(Fig. 4 A); these quickly became convulsive, while, at the same time the Rs became highly irregular; then followed bursts of TRs with corresponding types of respirations.

Atropine, 3 mgm. intravenously, at first enlarged the TRs, then abolished them; at the same time the atropine abolished the respiratory changes, inducing the large, regular respirations characteristic of this drug (Fig. 4 B). Following this atropinization 0.5 mgm. eserine intravenously and ACh 1 : 5 × 10^4 on the medullary floor failed to yield TRs and did not induce the usual respiratory changes. Thus atropine annuls the effects of eserine–ACh and precludes the usual actions of these substances.

The effects of the drugs are more evident in Figs. 5 and 6, in which a faster recording surface was used. Eight seconds after the injection of 0.5 mgm. eserine into the external jugular vein, humps (Hs) developed during expirations (Fig. 5 A); the humps changed into extra respirations (ERs), which soon came to exceed the original respirations (ORs); these latter decreased in amplitude and became of shorter period; these changes resulted in approximate doubling of the respiratory rate (Fig. 5 A). Tongue retractions (TRs) appeared immediately after the Hs and quickly augmented; observations of the levers indicated that each TR preceded slightly an OR and it seems likely that it was caused by overflow of the discharge from the inspiratory center; this will be explained under "Discussion".

ACh 1 : 5 × 10^7 applied to the medullary floor, intensified the respiratory effects and augmented the TRs, thus, as in the previous experiment, enhancing the effects of eserine (Fig. 5 B). The respiratory and lingual effects then declined in the reverse order of their development: thus the ERs declined, each again becoming a hump (H) on the expiratory descent; the ORs increased in extent, while remaining of brief period (Fig. 5 B, C). The TRs then declined, while still corresponding in rate to the ORs; finally the TRs became small oscillations at the same rate as the ORs (Fig. 5 C). Twenty-two minutes after intravenous eserine the ORs still showed the quickening and slight humps characteristic of the drugs; the corresponding TRs were then but slight oscillations (Fig. 5 C).

A second intravenous injection of 0.5 mgm. eserine again yielded typical effects, manifested by quickening of the Rs with humps (Hs), which this time did not develop into ERs; again the development of large TRs coincided with that of the Hs (Fig. 6 A). The medullary application of ACh 1 : 5 × 10^6 then quickened and augmented the Rs, accentuating the Hs; correspondingly the TRs enlarged greatly, as usual maintaining the respiratory rate (Fig. 6 A). In this preparation direct stimulation of the XII nuclei, with convulsive discharges, did not result from the concentrations of ACh used and further applications of ACh were not made. Instead a test was made with intravenous atropine, 4 mgm., to determine its effect at this particular stage: it was observed to augment the Rs and eliminate the Hs; the TRs first declined and then, in correspondence to the large Rs, enlarged briefly; finally the TRs

PLATE III

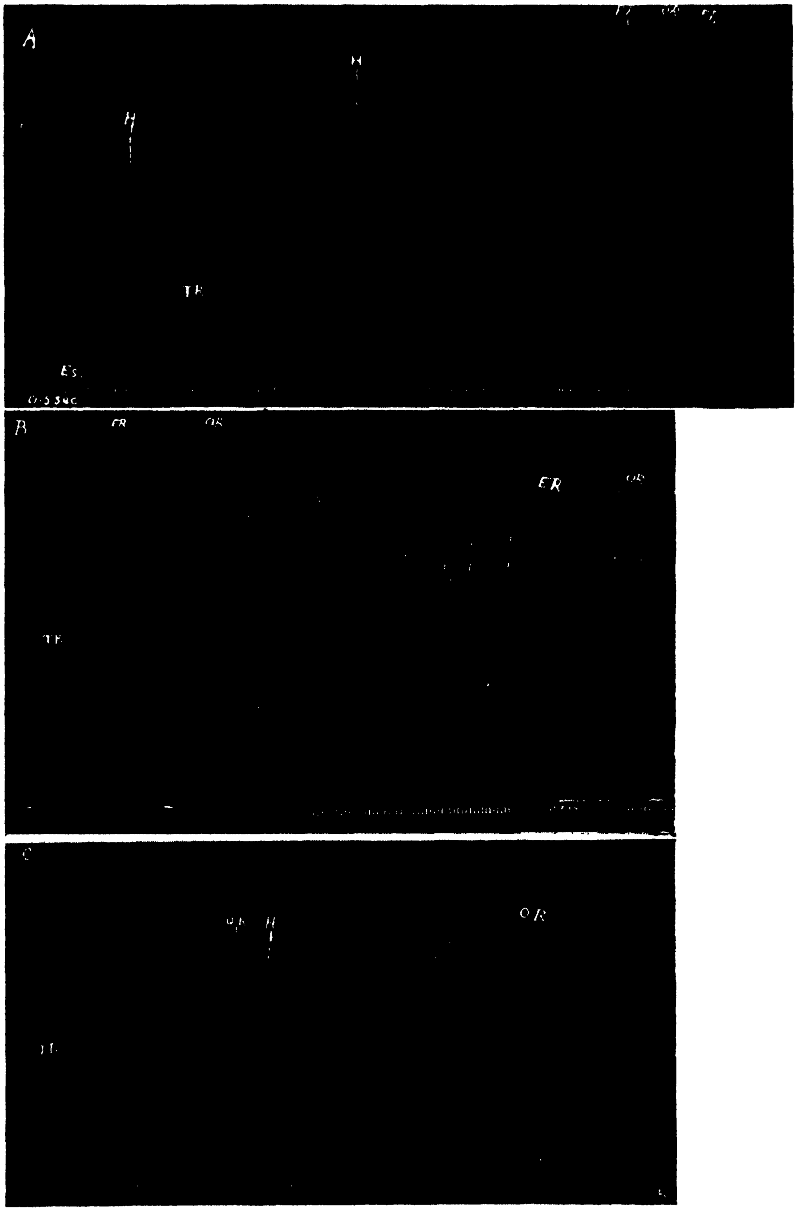

FIG. 5. *A. At signal 0.5 mgm. eserine into external jugular vein. B. 20 sec. before this record rectangle with ACh 1 : 5 × 10^7 on medullary floor. C. TRs continued after ERs have disappeared.*

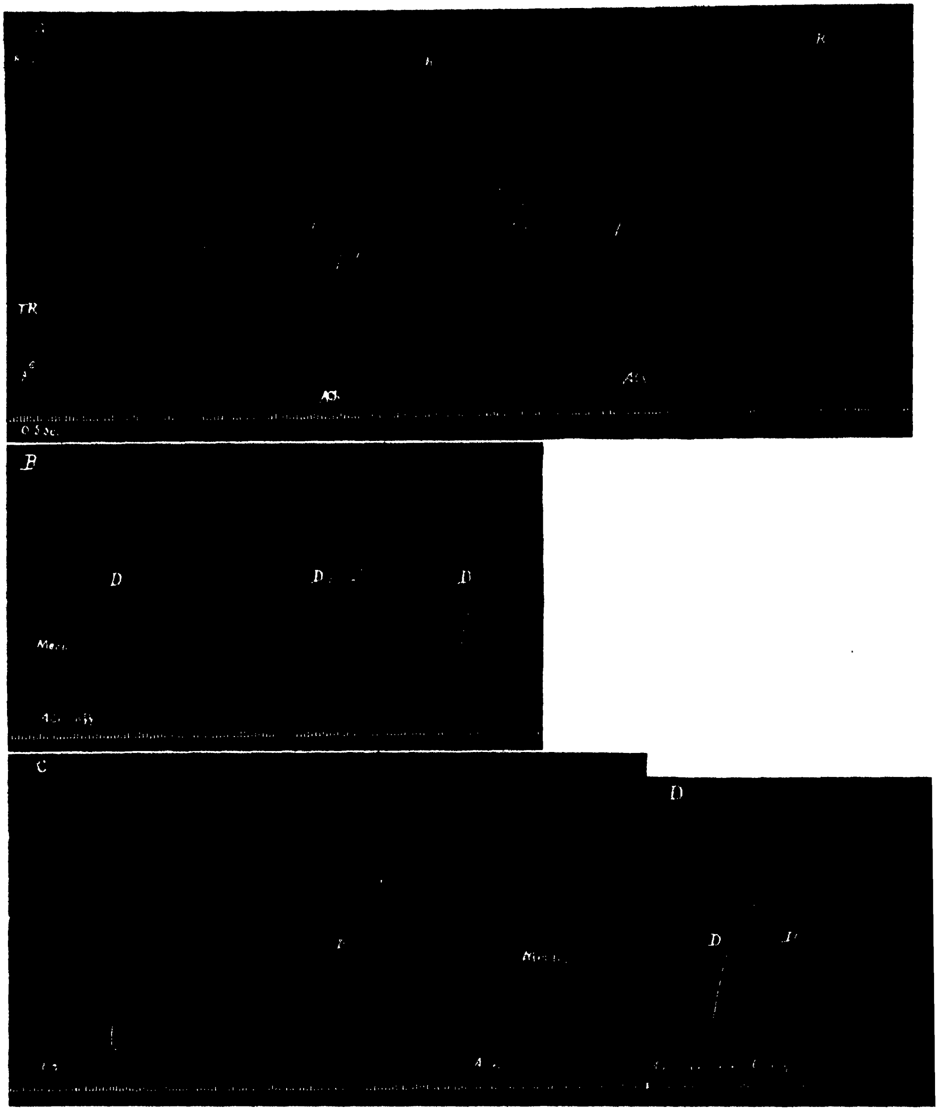

FIG. 6. *A. Just before this record 0.5 mgm. eserine intravenously. At first signal rectangle with ACh 1 : 5 × 10^6 on medullary floor: augmentation of TRs. At second signal 4 mgm. atropine intravenously: Rs augmented; Hs and TRs eliminated. B. D, postatropine deglutitions. At signal last ACh rectangle off. Mech., mechanical stimulation of nucleus XII by paper rectangle. C. At first signal 0.5 mgm. eserine intravenously: no effect. At second signal rectangle with ACh 1 : 5 × 10^6 on medulla: typical action of ACh precluded by atropine. Mech., mechanical stimulation of XII nucleus by paper rectangle. D. Saline applied to tongue base induced deglutitions, D: reflex arcs for deglutition functional after atropine.*

PLATE V

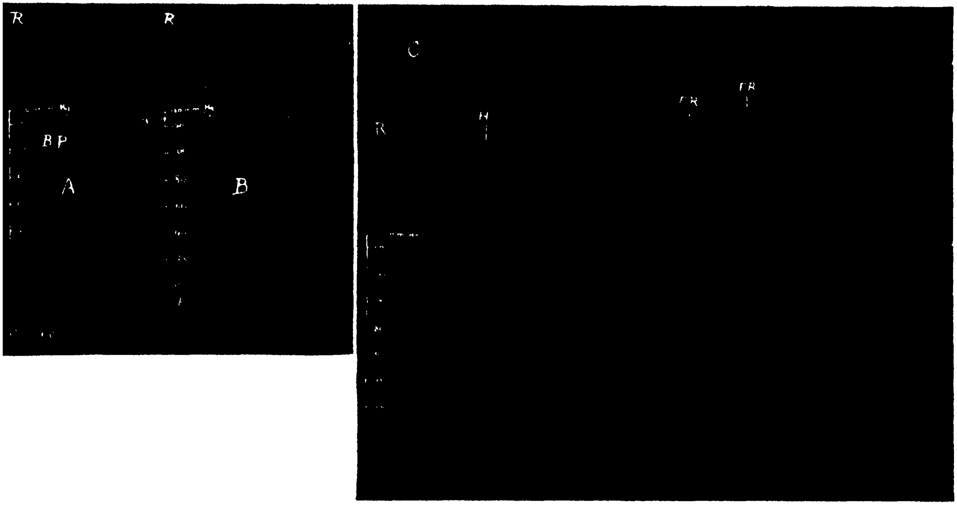

FIG. 7. *Upper record, respirations, R; lower record, B.P. A. Before eserine. B. At signal 0.4 mgm. eserine intravenously (proportionate dose for this animal): amplitude of Rs increased. C. Hs developed and became ERs. Cardiac inhibition; slight fall of B.P. changing into rise.*

PLATE VI

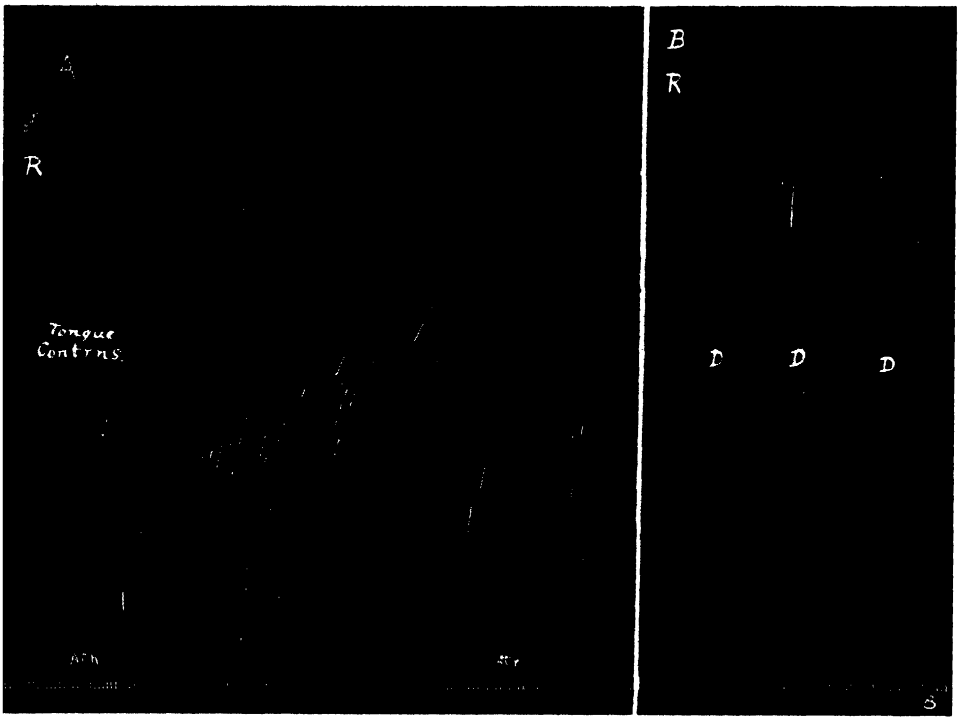

FIG. 8. *A. Prior to record, 0.5 mgm. eserine intravenously. Rectangles with ACh 1 : 5 × 10^7 applied three times to medullary floor; also rectangle with ACh 1 : 10^6 applied once. At first signal rectangle with ACh 1 : 5 × 10^7 to medullary floor: convulsive tongue contractions with deglutitions. At second signal 4 mgm. atropine intravenously: convulsive contractions abolished. B. Rs regular. D, postatropine deglutitions.*

disappeared (Fig. 6 A). There followed a series of deglutitions (Ds), which are usual after atropinization and appear to be characteristic of it (Fig. 6 B). Thereafter, intravenous eserine and medullary ACh 1 : 5 × 10^6 did not yield Hs nor other respiratory changes nor TRs (Fig. 6 C); these effects are thus precluded by atropine.

Saline applied to the tongue now induced deglutition (Fig. 6 D), from which it may be concluded that atropine does not paralyze the arcs involved in the deglutition reflex; it merely removes the eserine–ACh influences.

In order to exclude the possibility that a fall in blood pressure might contribute to the respiratory stimulation by eserine the effects of the usual dose of eserine were examined on respirations and blood pressure recorded simultaneously (Fig. 7). In 13 sec. after 0.4 mgm. eserine intravenously (the proportionate dose for this animal) the amplitude of the respirations was increased and in one minute 10 sec. later respiratory humps (Hs) appeared and, at the same time, definite cardiac inhibition; a few seconds later the blood pressure, which was 122 mm. before eserine, fell to 110 mm.; thereafter it rose to 128 mm. and then to 144 mm., coincidently with the development of extra respirations.

Therefore, it is apparent that the respiratory stimulation cannot be attributed to the very slight fall in blood pressure, particularly since the fall is converted into a rise, corresponding to the development of the very large extra respirations, resulting in almost doubling of the pre-eserine rate of respirations. Thus, in agreement with Gesell and Hansen (8), we may conclude that eserine stimulates the respiratory centers directly.

In the experiment illustrated in Figs. 5 and 6 the convulsive tongue activity did not develop from eserine–ACh as it did in the experiment shown in Figs. 2 to 4. The tongue activity was shown, however, in an intense form in Fig. 8. Here, following the procedures indicated in the legend, the medullary application of ACh 1 : 5 × 10^7 induced convulsive lingual activity; the convulsive contractions were very powerful, some of the movements recorded being probably deglutitions. At first the rate of the larger contractions corresponded roughly to that of the respirations but later the rate was much faster than that of the Rs and there was no correspondence between tongue contractions and Rs (Fig. 8 A).

Atropine intravenously, 4 mgm., abolished the lingual convulsions and then induced the characteristic outbursts of deglutitions (Fig. 8 B). Concurrently, the respirations changed from those characteristic of eserine–ACh to the large, regular, slow excursions typical of atropine. It was noted also in this experiment that atropinization precluded any further action of eserine (0.5 mgm.) or the medullary application of ACh 1 : 5 × 10^7 or 1 : 10^6. That the XII nucleus had, however, retained its excitability was shown by the fact that mechanical stimulation of the nucleus with a paper rectangle or small brush induced extensive tongue contractions.

In Fig. 9 A and B records are shown from a preparation that was unusually sensitive to ACh. Eserine, 0.5 mgm., had induced the usual respiratory quickening with extra respirations and TRs. Then ACh 1 : 5 × 10^7 on the medullary floor augmented the ERs and in one and one-half minutes induced convulsive contractions of the entire tongue (Fig. 9 A). Later ACh 1 : 10^7 immediately induced respiratory changes and tongue contractions with spreading of the tip (Fig. 9 B). Atropine, 4 mgm., suppressed the convulsive lingual activity, including tip spreading; however, a few rather extensive TRs followed atropine, later to be succeeded by very slight TRs corresponding to the respirations; they were due to slight contractions at the tongue base and resisted further atropinization.

It seems, therefore, that the neutralization of eserine–ACh by atropine is not always absolute, although it was noted that effects of eserine and ACh given subsequently were precluded. The usual postatropine deglutitions occurred, while the respirations became more regular. It was again observed that deglutition could be evoked by saline on the tongue, proving once more the integrity of the deglutition reflex arcs after atropine.

As already stated, it was regularly observed that intravenous eserine has a profoundly stimulating action on the respiratory centers; advantage was taken of this property in a preparation that had ceased breathing after decerebration and decerebellation. Respirations, which had been restored by 1.25 mgm. eserine, were of the usual eserine type, showing humps or extra respirations; the tongue showed a marked furrow with synchronous retraction, both together corresponding to a respiration (Fig. 10).

The dose of eserine, which was larger than usual, evidently resulted in very complete eserinization and rendered the responses to medullary ACh prompter and more intense: thus ACh 1 : 5 × 10^7 evoked, in one and one-quarter minutes, convulsive tongue movements (Fig. 10 A); the same concentration applied a second time evoked in three-quarters of a minute more powerful movements (not shown in Fig. 10); a third similar application evoked in one minute 9 sec. still more powerful movements accompanied by deglutitions (Fig. 10 B). Atropine, 4 mgm., intravenously, abolished these large movements, leaving a few tongue retractions, which quickly subsided (Fig. 10 B); however, a much reduced furrow at the tongue base, corresponding to each respiration was visible and indicated again the persistence of some eserine–ACh effects after atropinization. Postatropine deglutitions occurred as usual; the respiratory humps and extra respirations disappeared, while the original respirations became deep and regular.

Discussion

As was explained in the "Introduction", this research was directed primarily to determine the effects of ACh when applied in low concentrations to the floor of the fourth ventricle in the eserinized, decerebrated cat. The following discussion will be rendered more intelligible by a short statement regarding

PLATE VII

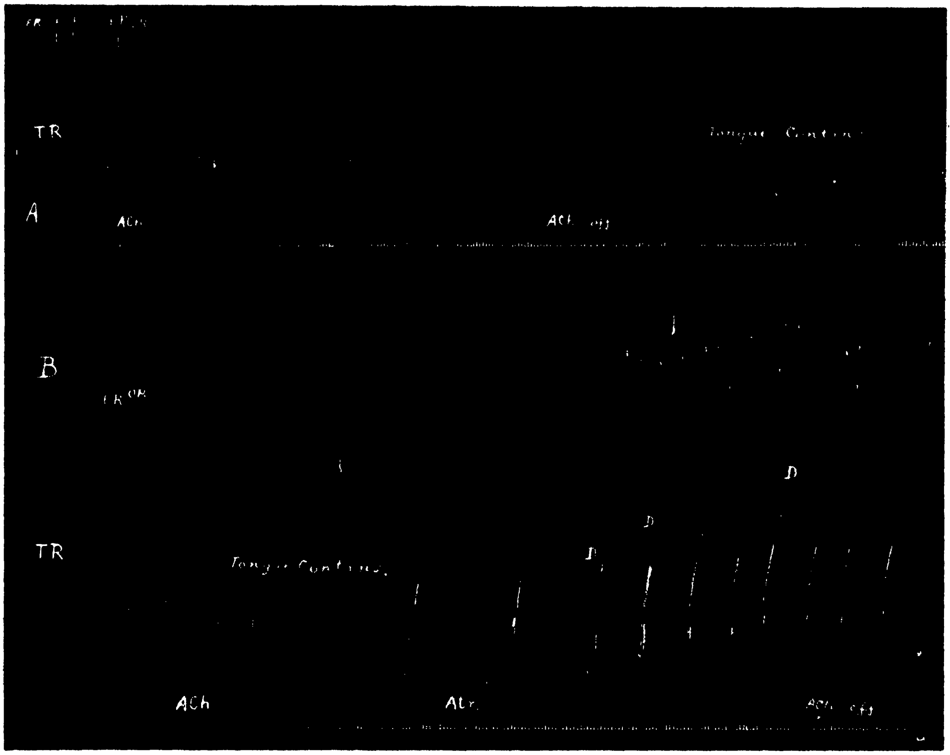

FIG. 9. *A. Prior to record, 0.5 mgm. eserine intravenously. At first signal rectangle with ACh* $1 : 5 \times 10^7$ *on medullary floor: ERs augmented, then convulsive contractions of entire tongue. At second signal ACh off. B. Between A and B rectangles with ACh* $1 : 5 \times 10^7$ *and* $1 : 5 \times 10^6$ *on medullary floor, each of which induced tongue contractions. At first signal rectangle with ACh* $1 : 10^7$ *on medullary floor, which induced respiratory changes and convulsive contractions of entire tongue, with repeated spreading of tip. At second signal 4 mgm. atropine intravenously: tongue convulsions suppressed; few TRs gradually decreasing. D, postatropine deglutitions. At third signal ACh off medulla. The tongue contractions in this experiment differed somewhat in character from those in other experiments: there was less retraction and more transverse and vertical contraction, with marked spreading of tongue tip. These movements, involving transverse and vertical intrinsic lingual muscle fibers, were less effective on the tongue lever, although very pronounced to visual inspection.*

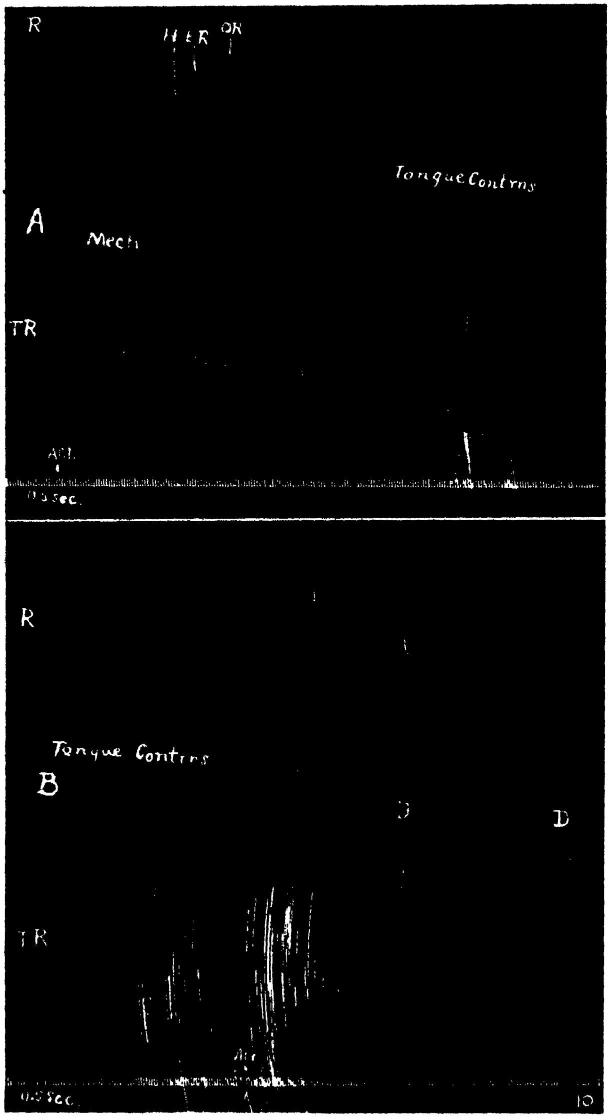

FIG. 10. *A. Eserine, 1.25 mgm. intravenously prior to record, had restored breathing. At first signal rectangle with ACh 1 : 5 × 10^7 on medullary floor: convulsive tongue contractions. Mech., due to mechanical stimulation of XII nucleus by application of rectangle. B. Rectangle with ACh 1 : 5 × 10^7 applied to medullary floor just prior to this record evoked, in one minute 9 sec., more extensive convulsive tongue contractions. At signal during contractions 4 mgm. atropine intravenously: contractions suppressed; few TRs gradually decreasing. D, postatropine deglutitions.*

the actions of eserine and ACh, which have been inferred from the results in this paper and earlier papers (Langley and Kato (9); Miller (10, 11, 12, 13, 14, 15); Miller, Stavraky, and Woonton (17)).

There is a considerable degree of similarity between the effects of intravenous eserine and of ACh when applied in low concentrations to the cerebral cortex and the floor of the fourth ventricle. It has been inferred that both drugs stimulate certain central synapses and facilitate conduction at these junctions. Preliminary eserinization was employed in all my experiments and, since the effects are striking, they will be considered first. It is known from the work of Worzniak and Gesell (21) that intravenous eserine induces hyperpnoea and that ACh on the medullary floor intensifies this effect; according to a verbal statement by Gesell, these effects are abolished by atropine. Detailed experiments by Gesell and Hansen (8) prove that eserine and ACh, the latter by intracarotid injection, stimulate the respiratory centers; also that the effects of both drugs are suppressed by atropine.

With these basic results of Gesell and his collaborators, my own are in agreement; however, their observations were made in the dog, while mine were made in the decerebrate cat; also in my experiments the Sherrington pneumograph was used and a complete study was made of the responses of the hypoglossal nucleus, as evidenced by contractions of the tongue muscles. In general it may be regarded as established that eserine and ACh stimulate the respiratory centers and that both drugs are opposed by atropine.

It is necessary first to discuss the effects of preliminary intravenous eserine and subsequently those resulting from the medullary application of ACh after eserinization. The observations recorded in this paper show that intravenous eserine in a dose of 0.15 mgm. per kgm. of body weight in the cat augments or accelerates the respirations; later "humps" (Hs) appear and develop into extra respirations (ERs). These changes cannot be attributed to the accompanying fall in blood pressure, which is very slight and brief (Fig. 7), being converted into a rise, coincident with the appearance of extra respirations. Consequently it may be concluded that intravenous eserine exerts a direct stimulating action on the respiratory centers; this conclusion is in harmony with the inference of Gesell and Hansen that respiratory stimulation by eserine is due to direct central nervous action (8).

Since the extra respirations induced by eserine are in reality extra inspirations it appears that the stimulating action of eserine is exerted on the inspiratory centers, of both sides, described by Pitts, Magoun, and Ranson (18); each center is situated in the ventral reticular formation over the cephalic four-fifths of the inferior olive. In Fig. 11 the suggested modes of action of eserine and ACh are illustrated. It may be supposed that eserine exerts its stimulating effect by acting on synapses of the type of S.1, this being the termination of an axon of unspecified origin; thus excitation of the center would be initiated.

We may now consider the likelihood that eserine facilitates irradiation throughout the inspiratory center. The basis for this action is provided in the conclusion of Pitts *et al.* (19) that the constituent neurones are connected together by extensive synapses, so that irradiation over the entire center readily occurs, when a small area is stimulated. Now, reasons have been given in a previous paper (10), as well as in the present paper, for believing that eserine facilitates synaptic transmission and this property would lead to irradiation of the initial eserine excitation within the inspiratory center, as illustrated in Fig. 11: here, inspiratory neurone I.N.1 is excited by eserine acting on synapse S.1; the excitation overflows through a facilitated pathway by a recurrent collateral (R.C. 1) and an internuncial neurone to I.N. 2; thence by a similar facilitated pathway (R.C. 2) to I.N. 3; in each case the connecting synaptic pathway is facilitated by the eserine. Thus discharges of the three inspiratory neurones will be practically synchronized and this process will result in the sharp, snappy inspirations often seen in the records (OR, Fig. 9).

This process of neuronal synchronization may lead to increased amplitude of the original respirations (ORs, Fig. 9); on the other hand, the interpolation of extra respirations (ERs), due to general excitation of the inspiratory neurones by eserine, may, at times, reduce the amplitude of the ORs, although they are of very short period (Fig. 5).

The extra respiration may be compared, in a general way, to the extra systole of the frog's heart, as evoked by a stimulus following the refractory period. Regarding time relations, the ER may occur during expiration (Fig. 5) but it may occur also during inspiration (Fig. 2) and thus its precise relation to the respiratory cycle or to any possible refractory phase of that cycle has not yet been determined. In general, it appears that the extra respiration reflects the hyperexcitability of the inspiratory neurones under the influence of eserine, the extra inspiratory impulse being generated at a relatively excitable period of the respiratory cycle.

The irradiation from the inspiratory centers to the XII nuclei, as induced by eserine, will next be considered. That eserine should *create* pathways for this irradiation seems improbable on general grounds; rather would eserine facilitate conduction over pre-existing pathways. Now it was pointed out under "Results" that a moderate amount of irradiation of this kind may occur normally (as in the panting dog) and without the use of eserine and that, therefore, potential pathways to subserve the irradiation process must be supposed to be present. Possible pathways are illustrated in Fig. 11: recurrent collaterals (R.C.3,4) from inspiratory neurones convey overflow inspiratory impulses through internuncials to the XII nuclei. Thus eserine merely intensifies conduction over such pathways, the prior existence of which must be assumed.

It has already been explained how the inspiratory neurones are synchronized by eserine; consequently the impulses over the various components of the above irradiation pathways will likewise be synchronized. Hence the motoneurones of both XII nuclei will be bombarded by these impulses until C.E.S. will lead to a discharge in response to each excitatory volley; thus the discharges from the XII nuclei will occur at exactly the same rate as those from the inspiratory neurones proceeding to the inspiratory muscles.

An additional element in causing the irradiation from the inspiratory centers to the XII nuclei is to be found in the property of eserine, already mentioned, of facilitating synaptic transmission. In this way conduction through the facilitated irradiation pathway (Fig. 11) will be rendered readier for the synchronized overflow impulses from the inspiratory neurones.

It will be recalled that the tongue retraction (TR) precedes slightly the corresponding original respiration (OR), which is actually an inspiration and induces the TR by overflow. This slight precedence, which may be observed before the use of eserine, was explained under "Results", namely by the shorter conduction pathways from inspiratory centers to XII nuclei to tongue muscles as compared to those from inspiratory centers to spinal motoneurones to intercostal muscles. This explanation is valid also after the use of eserine, only that in this latter case the irradiation process is much more intense. The muscles involved in the tongue retractions are the hyoglossi and styloglossi, together with the intrinsic longitudinal muscle fibers of the tongue.

Consideration will now be given to the effects of applications of low concentrations of ACh to the floor of the fourth ventricle, following preliminary eserinization. As described under "Results" these effects are: First to increase hyperpnoea and irradiation from inspiratory centers to XII nuclei; with respect to the hyperpnoeic effect, my results agree with those of Worzniak and Gesell (21), though they did not report the irradiation, nor did they mention the concentrations of ACh used. In my own experiments the above effects were secured by the application of ACh concentrations of $1 : 5 \times 10^7$ to $1 : 10^6$. The hyperpnoea and irradiation to the XII nuclei, when induced by eserine, subside after a time; medullary ACh then often restores both reactions (Fig. 3); this duplication by ACh of the prior effects of eserine was observed repeatedly and indicates a similar mode of action of both substances; thus support is provided for a basic thesis of this work, namely that each substance stimulates and facilitates certain central synapses.

It must be supposed that ACh applied to the floor of the fourth ventricle diffuses ventrally through the more dorsally situated expiratory centers (18) to reach the inspiratory centers, which it stimulates; any possible effect of ACh on the expiratory centers during its course was not detected and, in any case, must be slight. In stimulating the inspiratory centers, ACh may be supposed, like eserine, to excite synapses of the type of S.1, Fig. 11; ACh would also facilitate synaptic transmission between the inspiratory neurones; finally it would facilitate synaptic transmission over the irradiation pathway to the XII nuclei (Fig. 11). These effects of medullary ACh thus reproduce, in general, those of intravenous eserine.

The second response to medullary ACh is not evoked, in my experience, by intravenous eserine alone: following eserinization, medullary ACh, in concentrations varying from $1 : 5 \times 10^7$ to $1 : 5 \times 10^4$, frequently evokes powerful discharges from the XII nuclei, as shown by the convulsive contractions of the lingual muscles; the effective concentration of ACh varies with the degree of preliminary eserinization and the susceptibility of the preparation (Figs. 4, 8 A, 9, 10).

In explaining this powerful stimulation of the XII nucleus we may recall that ACh stimulates various central synapses. Now, Cajal has described pericellular plexuses around the neurones of the XII nucleus (3) and these plexuses must be regarded as terminating in end feet on the neurones. Hence, it is logical to infer that ACh stimulates these synapses, thus evoking convulsive discharges of the XII nucleus, which in turn induce powerful contractions of the lingual muscles; the synapses involved are shown in Fig. 11.

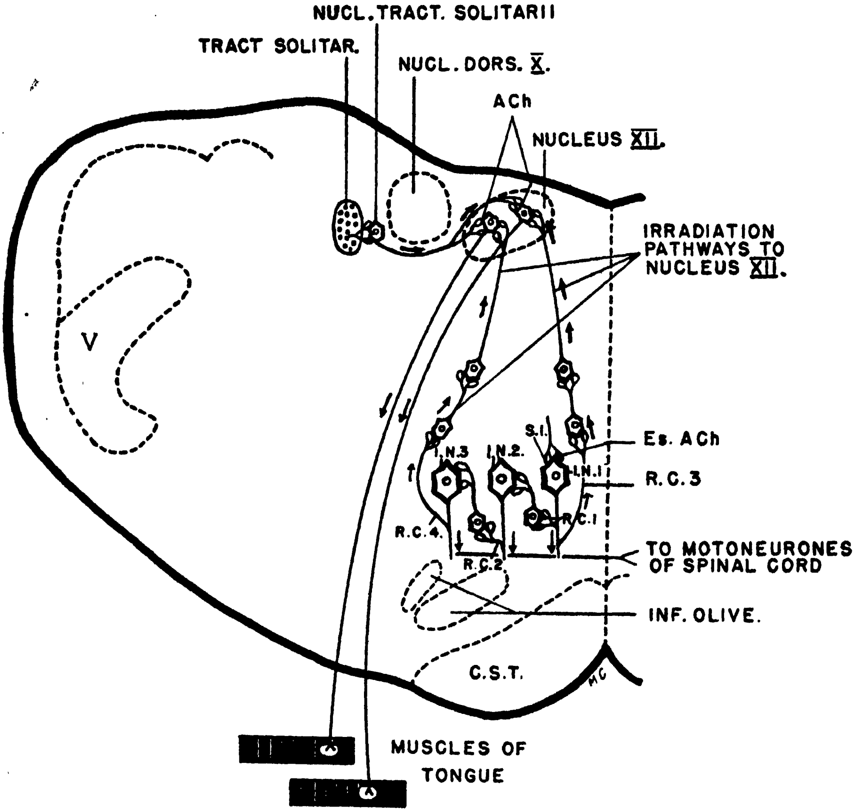

FIG. 11. *Section of medulla oblongata of cat, semidiagrammatic. Eserine and ACh stimulate inspiratory neurones by acting on synapse S.1, the termination of an axon of unspecified origin. Inspiratory neurones, I.N.1, I.N.2, and I.N.3 are synchronized by recurrent collaterals R.C.1, R.C.2, and internuncials. Irradiation from inspiratory center to nucleus XII is effected through recurrent collaterals, R.C.3, R.C.4, and internuncials. ACh acting on synapses surrounding neurones of nucleus XII induces convulsive tongue contractions. Part of pathway for reflex deglutition is from tractus solitarius (Tract. solitar.) to nucleus XII. For further details see text.*

Quite comparable to the actions of ACh on the hypoglossal nucleus are those of this substance on the spinal motoneurones reported by Bülbring and

Burn (2). These workers used two separate blood perfusing systems, one for the lower spinal cord, the other for the muscles of the hind leg. Following eserinization 1 μgm. of ACh injected into the cord circulation was effective in evoking a series of contractions of the tibialis anterior; without eserinization a much larger dose of ACh was necessary for stimulation of the spinal motoneurones. Bülbring and Burn found further that this spinal excitation by ACh is counteracted by atropine, just as is the excitation of the XII nucleus in my own experiments.

Thus medullary ACh induces stimulation of the synapses around the motoneurones of the XII nucleus, a reaction that was not induced by intravenous eserine alone. While admitting this divergence, it is concluded that both eserine and ACh stimulate various synapses and facilitate transmission at these junctions.

The high degree of similarity between the actions of eserine and ACh leads one to infer that many of the actions of eserine are dependent on its anticholinesterase property, thus leading to the accumulation of "natural" ACh, which may be regarded as the effective agent at the synapses. But whether, in addition, eserine exerts a specific synaptic action cannot be decided on the evidence at present available.

It might appear that the effects of ACh described in this paper should be classed as "muscarine" actions (Dale (4)), since they are almost completely suppressed by atropine. However, Dale and Gaddum (7) showed that the "nicotine-like" response to ACh of an isolated strip of denervated diaphragm is precluded by atropine. Also Abdon (1) reported that the nicotine-like "quick contraction" of the rabbit's gastrocnemius requires a large amount of ACh for its induction and likewise a large amount of atropine for its abolition. Thus it appears that the distinction between muscarine-like and nicotine-like effects of ACh is not absolute. However, since my own results were obtained with minute amounts of ACh and were suppressed with comparatively small amounts of atropine (4 mgm. in a 3 kgm. cat) they show rather more affinity with the muscarine-like than with the nicotine-like actions of ACh.

As just stated, Bülbring and Burn (2) established the antagonism of atropine towards eserine and ACh, when acting on spinal motoneurones. The same antagonism was emphasized, in the case of the respiratory center, by Gesell and Hansen (8), who wrote:

> "We are forced to believe, contrary to generally accepted opinion, that atropine is capable of exercising a powerful anti-acetylcholine and anti-eserine action in the nerve cells of the central nervous system. Our findings and those of Miller, Stavraky, and Woonton [17] corroborate those of Marrazzi in peripheral ganglia and make the observation general for all nerve cells".

While the authors cited regard the stimulating actions of eserine and ACh as being exerted, in a general way, on the neurones, my own inferences lead me to believe that these substances stimulate and also facilitate the synaptic junctions of these cells. The antagonism of atropine towards eserine and ACh, as recorded in the present paper, is in complete harmony with the findings of Bülbring and Burn and of Gesell and Hansen.

It is well known that Dale (5) suggested that ACh might act as transmitter at certain central synapses. In a recent presentation (6) Dale reiterates this suggestion, together with the further speculation (if I understand him correctly) that nor-adrenaline and possibly histamine may be functionally involved at other varieties of synapses. In the present paper evidence is furnished for the conception that ACh stimulates and facilitates certain central synapses; this conception is compatible with the suggestion of Dale that natural ACh may play an important role at some types of synapses of the central nervous system.

Acknowledgment

The author wishes to express his thanks to Dr. Murray L. Barr for advice in the preparation of Fig. 11.

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